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Journal of Chromatography B, 717 (1998) 201–245

JOURNAL OF CHROMATOGRAPHY B

Review

Application of gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry to assess in vivo synthesis of prostaglandins, thromboxane, leukotrienes, isoprostanes and related compounds in humans[1]

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Abstract

Prostaglandins, thromboxane, leukotrienes, isoprostanes and other arachidonic acid metabolites are structurally closely related, potent, biologically active compounds. One of the most challenging tasks in eicosanoids research has been to define the role of the various eicosanoids in human health and disease, and to monitor the effects of drugs on the in vivo synthesis of these lipid mediators in man. Great advances in instrumentation and ionization techniques, in particular the development of tandem mass spectrometry and negative-ion chemical ionization (NICI), in gas chromatography and also advances in methodologies for solid-phase extraction and sample purification by thin-layer chromatography and high-performance liquid chromatography have been made. Now gas chromatography–mass spectrometry (GC–MS) and GC–tandem MS in the NICI mode are currently indispensable analytical tools for reliable routine quantitation of eicosanoid formation in vivo in humans. In this article analytical methods for eicosanoids based on GC–MS and GC–tandem MS are reviewed emphasizing the quantitative measurement of specific index metabolites in human urine and its importance in clinical studies in man. Aspects of method validation and quality control are also discussed.

Keywords: Reviews; Prostaglandins; Thromboxane; Leukotrienes; Isoprostanes

Contents

[1] Dedicated to Prof. Jürgen C. Frölich for his 60th anniversary.

0378-4347/98/$19.00
PII: S0378-4347(98)00210-2

1. Introduction

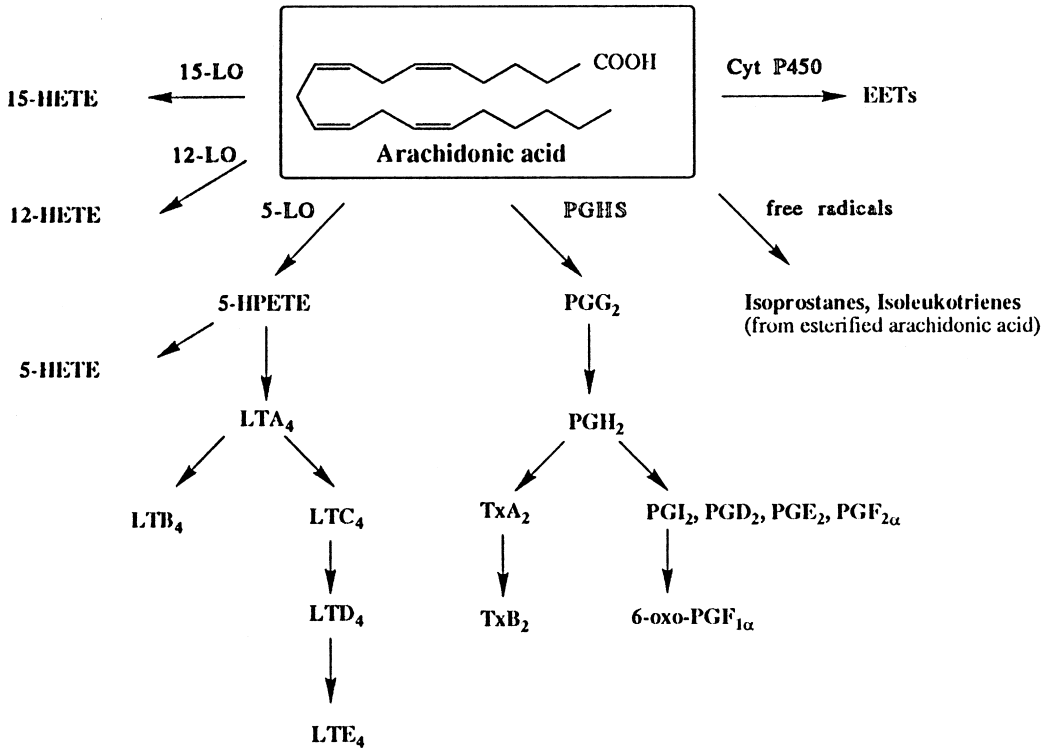
Mass spectrometry in combination with gas chromatography (GC–MS) has been the key analytical approach used for the structural identification of prostaglandins, thromboxanes, leukotrienes, isoprostanes and numerous other chemically closely related, arachidonic acid-derived compounds in biological matrices, for the ascertainment of their metabolic fate in humans and for the determination of specific circulating and urinary index metabolites. Measurement of specific index metabolites of these eicosanoids is a useful approach to assess in vivo formation of these compounds and allows the effects of diseases and drugs on their synthesis to be monitored. In the beginning of eicosanoid research, GC–MS in the electron impact (EI) ionization mode was the analytical method of choice for the quantitative determination of index metabolites in plasma and urine of humans. Furthermore, GC–MS has been repeatedly used to validate alternative methods based on radioimmunoassay (RIA) and enzyme-immunoassay (EIA) which have also been frequently used for the measurement of eicosanoids.

Methods based on GC–MS consist of several analytical steps required for extraction, separation, purification, derivatization and finally gas chromatographic separation followed by mass spectrometric detection. In the last two decades, considerable improvements in ionization techniques, i.e. the development of negative-ion chemical ionization (NICI), in mass spectrometry, in particular the development of gas chromatography tandem mass spectrometry (GC–tandem MS), and ultimately in the field of other chromatographic separation techniques, such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and solid-phase extraction (SPE) have been achieved. Thanks to these improvements, specific, accurate and routine measurement of eicosanoid formation in vivo in man is currently possible in only a few ml of urine or plasma by GC–MS and in particular by GC–tandem MS. At present, methods based on GC–MS and GC–tandem MS are indispensable analytical tools to reliably assess eicosanoid formation in vivo in man. This paper critically reviews the most significant GC–MS and GC–tandem MS methods which have been used to assess in vivo synthesis of prostaglandins, thromboxane, leukotrienes, isoprostanes and related compounds in humans. Special emphasis was given to the quantitative measurement of specific urinary and circulating index metabolites for each class of the eicosanoids. The single components of GC–MS methods including sample storage, use of internal standards, extraction, chromatographic separation, derivatization and final detection by mass spectrometry are thoroughly discussed. The importance of GC–MS in clinical studies in man and aspects of method validation and quality control are also discussed in this article.

2. Formation, metabolism and biological effects of eicosanoids

Arachidonic acid, 5,8,11,14-*cis*-eicosatetraenoic acid, is the major C_{20}-polyunsaturated fatty acid in most mammalian systems. Upon various stimuli, arachidonic acid is released from the esterified stores of phospholipids by the action of phospholipases. Once released, arachidonic acid is metabolized to a large family of oxygenated C_{20}-fatty acids via distinct enzymatic and nonenzymatic metabolic pathways (Scheme 1). The members of this family, collectively termed eicosanoids [1], are prostaglandins (PGs) and thromboxanes (Txs) formed via the cyclooxygenase (COX) pathway, 5-hydroxyeicosatetraenoic acid (5-HETE), leukotriene (LT) B_4 (LTB_4), and the cysteinyl leukotriene C_4 (LTC_4) formed via the 5-lipoxygenase (LO) pathway, as well as 12-HETE and 15-HETE and lipoxins (not shown) formed via the 12- and 15-LO pathway, respectively. Thromboxane A_2 (TxA_2) and prostacyclin (PGI_2) are highly unstable and are nonenzymatically converted to TxB_2 and 6-oxo-$PGF_{1\alpha}$, respectively. LTC_4 is metabolized to LTD_4 and LTE_4 by successive elimination of a γ-glutamyl residue and glycine. LTC_4, LTD_4, and LTE_4 are known as the 'slow reacting substance of anaphylaxis' (SRS-A). Cytochrome P-450 epoxygenases catalyse the conversion of arachidonic acid to epoxyeicosatrienoic acids (EETs). In addition, arachidonic acid is susceptible to autooxidation yielding various hydroxyperoxyeicosatetraenoic (HPETE) acids and the newly discovered isoprostanes and isoleukotrienes.

Eicosanoids are formed by virtually every tissue in the body and are widely distributed. They act locally. Only very small amounts are necessary to elicit biological responses. The biological activities of eicosanoids are of great physiological and pathophysiological importance. The most important biological effects of eicosanoids include control of vascular tone, renal function, platelet aggregation,



Scheme 1. Schematic diagram of the main metabolic pathways of arachidonic acid. LO, lipoxygenase; PGHS, prostaglandin H synthase; Cyt P450, cytochrome P450. More details are given in Section 2.

hypersensitivity and inflammation. Primary eicosanoids are inactivated rapidly by specific enzymes at the site of origin, in the lung or in the liver, to numerous chemically closely related substances. The most relevant catabolism involves oxidation of the 15-hydroxy group of prostanoids to yield the 15-oxo-metabolites, β-oxidation, ω-oxidation, and ω-oxidation followed by β-oxidation to yield more polar dicarboxylic acids. The molecular biology and enzymology of eicosanoids, the regulatory mechanisms in eicosanoid release and metabolism, their biological effects, and their role in immune reactions, cancer and inflammatory processes have been thoroughly reviewed elsewhere [2–19].

3. Analysis of eicosanoids

Since the discovery and elucidation of the structure of prostaglandins, thromboxanes, leukotrienes, other eicosanoids and their metabolites, numerous analytical techniques have been developed for their analysis in biological samples. These include GC–MS using different ionization techniques (see Sections 4 and 5), liquid chromatography (LC) in combination with UV [20–35], fluorescence [36,37], or electrochemical detection [38], and more recently LC combined with mass spectrometry utilizing different ionization techniques [39–49], and also electrophoretic techniques [50,51]. Besides these physicochemical methods, bioassays and approaches based on RIA and EIA have also been developed [48,52–78].

Analysis of eicosanoids in vitro in studies on isolated cells and organs in order to explore basic mechanisms of formation and action of eicosanoids has been relatively easy to perform. Reliable analysis of eicosanoids in vivo, however, is a difficult and challenging analytical undertaking. Problems and strategies concerning the quantitation of eicosanoid formation in vivo have been the matter of many excellent reviews [54,79–100]. Most difficulties in eicosanoids analysis stem essentially from the occurrence of numerous chemically closely related compounds in human plasma and urine at very low concentrations. This requires the use of selective and highly sensitive physicochemical or immunological methods including a series of extraction and separation procedures. Another problem has been the definition of specific index metabolites and the search for suitable biological matrices. Monitoring circulating metabolites in whole blood or plasma is one approach to measure in vivo production, but there is always a risk of artefactual ex vivo formation during blood sampling [72,101,102]. Urine, on the other hand, is a biological fluid that is easy to collect without any such significant risk. Today, it is well established that quantification of circulating or urinary eicosanoids metabolites represents a more reliable approach of assessing endogenous eicosanoid synthesis in vivo than does quantification of the primary compounds [80,98,103]. The pattern of metabolites in urine most likely reflects the whole body production. However, it is known that intact urinary PGE_2 and TxB_2 may originate from the kidneys. Simultaneous measurement of primary eicosanoids and their metabolites in human urine permits assessment of both renal and systemic eicosanoid production. Urine has been found quite useful for measuring both whole body and renal production of eicosanoids.

3.1. Analysis by immunoassays and mass spectrometry

At present, immunoassays, i.e. RIA and EIA, and mass spectrometry, i.e. GC–MS are the most frequently used approaches for the quantitation of eicosanoid formation in vivo. Specific RIA and EIA are commercially available for many but not for all eicosanoids. These techniques, however, may lack specificity, particularly where complex biological fluids such as plasma and urine are analysed. Use of RIA and EIA methods for eicosanoid quantitation in plasma and urine requires their validation by GC–MS methods [52,56,78,104,105]. Currently, GC–MS is considered as the ultimate reference assay for most eicosanoids.

GC–MS has been extensively used for eicosanoid analysis, both for structure elucidation and quantitation. This technique offers the selectivity of GC separation utilizing the high resolution capacity and high thermal stability of chemically bonded fused-silica capillary columns coupled with the specificity and sensitivity of mass spectrometric analysis. Methodological and instrumental developments now make

it possible to reliably quantitate by GC–MS most eicosanoids in human plasma and urine at the low picogram per ml level.

The major drawback of methodologies based on GC is the absolute requirement for volatility and thermal stability of the analytes at the GC-operating temperature. This has, in the past, precluded the application of GC–MS to many polar or thermally labile eicosanoids such as cysteinyl leukotrienes. However, eicosanoid volatility is drastically increased by derivatization. Polarity and thermal lability have been almost completely overcome so that in principle, GC–MS is applicable to any eicosanoid including the highly polar, nonvolatile and thermally labile cysteinyl leukotrienes being catalytically reduced [106]. The desire to analyse any eicosanoid in its native form, i.e. without any derivatization, has led to the development of LC–MS methods and various ionization techniques such as thermospray, electrospray and particle beam ionization [39–49] that should, in theory, overcome many of the problems associated with GC–MS. Like HPLC separation and UV detection of LTs and HETEs, which have been widely employed in studies on isolated cell systems, the sensitivity of LC–MS detection, which is commonly in the low nanogram or upper picogram range, is not sufficient for the determination of endogenous concentrations of eicosanoids in human urine and plasma. Native eicosanoids have also been analysed by MS using ionization techniques such as desorption chemical ionization [107] or FAB [108]. However, these MS techniques have been used almost exclusively for structure elucidation.

3.2. Quantitative determination by GC–MS and GC–tandem MS

3.2.1. General remarks

Analytical methods for quantitative determination of eicosanoids in human urine and plasma based on GC–MS and GC–tandem MS consist of a series of procedures. They include extraction from a given biological sample of a particular endogenous eicosanoid and its stable isotope-labelled analog which has been added to the sample for use as internal standard, several derivatization and purification steps, and final GC separation followed by on-line MS analysis of GC peaks of the endogenous compound and the respective internal standard. Usually, GC is performed applying an oven temperature program starting at an initial temperature of 80°C to 100°C. Using oven temperature rates of 20°C/min to 30°C/min, a final temperature of 300°C to 320°C is achieved. Depending on the eicosanoid(s) to be analysed and on the GC column used, one GC–MS analysis takes about 20 to 40 min. GC–MS methods for cysteinyl leukotrienes additionally involve a treatment step for simultaneous catalytical reduction of their double bonds and desulphurisation of their thioether moiety to yield the corresponding 5-hydroxy fatty acid. Once the particular index metabolite, the biological sample, the sampling technique and sample storage have been defined, any of the above mentioned steps has to be optimized in order to achieve maximum sensitivity, specificity and accuracy. As a rule, methods involving quantification by GC–MS require more extensive sample purification, i.e. more analytical steps, than those applying GC–tandem MS so that quantitation of eicosanoids by GC–MS methods are substantially more laborious and time-consuming and often less accurate and specific than by GC–tandem MS methods.

For the last three decades, many groups dealing with the analysis of eicosanoids in biological fluids have developed sophisticated methods considering specific physicochemical properties of these compounds. The trend for the last years has consisted of the development of novel more simple methods and the improvement of already existing methods in terms of simplicity, routine analysis, i.e. high throughput and automation, versatility and effectiveness, i.e. simultaneous quantitation of many eicosanoids in a single biological sample within a single analytical procedure; also attempts have been undertaken to replace GC–tandem MS by the considerably cheaper GC–MS methodology [109–114].

Recently, COX-independent, free-radical catalysed prostanes, the isoprostanes, have been identified in human plasma and urine samples by GC–MS [115–117]. The existence of these compounds in biological fluids has been known as long as that of COX-dependent prostanoids [118]. For the quantitation of isoprostanes such as 8-iso-$PGF_{2\alpha}$, currently the best characterized F_2-isoprostane, GC–MS methods

based on already existing methods have been developed in recent years [91,119–123].

3.2.2. Index metabolites and biological matrix

The problems associated with the initial decision to index metabolites of eicosanoids and biological matrix have been reviewed elsewhere [82,86]. Specific index metabolites and biological matrices for eicosanoids reviewed in this article are thoroughly discussed in Section 4.

3.2.3. Internal standard

The use of internal standards for accurate and precise quantitative determination of eicosanoids by GC–MS is an absolute requirement. An 'ideal' internal standard has to have identical physicochemical properties with the respective endogenous eicosanoid throughout all the analytical steps of the method, i.e. extraction, derivatization and chromatography, with the sole exception of its molecular mass by which endogenous eicosanoid and the internal standard are discriminated by MS. As a rule, these criteria are fulfilled by synthetic eicosanoids in which sufficient atoms of ^{1}H, ^{12}C, or ^{16}O have been replaced by the corresponding stable isotopes ^{2}H, ^{13}C, or ^{18}O. When the internal standard is a stable-isotope-labelled analog of the analyte and is added to the matrix to be analysed, the method is known as isotope-dilution mass spectrometry (ID-MS). ^{2}H- and ^{13}C-labelled compounds are today commercially available for many eicosanoids in high isotopic purity. For some eicosanoids, stable-isotope-labelled analogs are not yet commercially available. For such eicosanoids, and in principle for any eicosanoid, chemical and enzymatic ^{18}O-labeling of carboxylic ^{16}O-atoms of commercially available eicosanoids is an easy procedure which is accessible to any laboratory [69,124–135]. Partial and total chemical synthesis of various ^{2}H- and ^{13}C-labelled eicosanoids have been described for many eicosanoids [79,108,136–149]. The main disadvantage of such synthetic routes is the necessity of special precursors which are not generally accessible. In the beginning of the use of GC–MS for quantitation of eicosanoids, the lack of suitable internal standards hampered accurate quantitation. For oxo-groups containing eicosanoids such as the major urinary metabolite of E prostaglandins, PGE-M, the use of already trideutero methoximated analogs has partially solved the lack of an internal standard but at the cost of inaccuracy and imprecision due to variations in the methoximation [150]. Generally, the use of partially derivatized eicosanoids as internal standards is problematic and not up-to-date in modern eicosanoid analysis.

Some precautions have to be taken into consideration concerning the use of stable isotope-labelled analogs as internal standards in quantitative GC–MS analysis of eicosanoids. These precautions include considerations of: (i) the isotopic impurity of the internal standard and of the choice of a proper amount of the internal standard to be added to the biological sample; (ii) the possibility of back-exchange of carboxylic-^{18}O by ^{16}O from water in esterase-rich matrices such as plasma [124,126]; (iii) the possibility of exchange of ^{2}H by ^{1}H during catalytical hydrogenation [151]; and (iv) the choice of the time of the addition of the internal standard. Synthetic stable isotope-labelled eicosanoids usually contain unlabelled material as isotopic impurity. The degree of this impurity has to be accurately determined. The concentration of the internal standard in a biological sample should be chosen in such a way that the contribution of the unlabelled material present in the preparation of the internal standard to the endogenous eicosanoid is negligible. Ideally, the added concentration of an internal standard in a certain biological sample should be of the order of the expected concentration of the corresponding eicosanoid. Regarding the choice of the concentration of the internal standard in a matrix, it must also to be taken into consideration that in some pathological conditions and in clinical studies with drugs, the in vivo formation of eicosanoids may be considerably altered. As a rule, the isotopic purity of an internal standard determines the range of linearity of a ID-MS method. Most of the commercially available ^{2}H- or ^{13}C-labelled eicosanoids contain considerably less than one percent of the unlabelled eicosanoids so that the range of linearity may be of three orders of magnitude. This wide range of linearity allows accurate quantitation of various eicosanoids in healthy and diseased humans under basal conditions and after administration of drugs. In the case of using carboxy-^{18}O-labelled eicosanoids

as internal standards, the readily and commonly occurring lactonization of eicosanoids urges special precautions. Since ^{18}O-labelled eicosanoids are prepared at a microgram quantity they have to be precisely standardized by using accurately weighed amounts of the starting unlabelled eicosanoid [124]. The extent of exchange of ^{2}H by ^{1}H during catalytical hydrogenation should be determined and considered in estimating endogenous levels of eicosanoids [151]. Regarding the time of addition, the internal standard should be added to the biological sample as soon as possible, preferably immediately after collection of the sample. In the case of using ^{18}O-labelled eicosanoids, esterase activity in esterase-rich matrices such as plasma should be inactivated prior to addition of the internal standard [124,126].

With respect to other chromatographic properties, native or derivatized labelled eicosanoids behave almost identically with their unlabelled analogs so that no considerable or total separation during SPE, HPLC or TLC occurs. Nevertheless, a significant difference in retention time between unlabelled and multi-tritium-labelled eicosanoids has been observed in the HPLC analysis of many eicosanoids [34]. During GC with fused-silica capillary columns, derivatized ^{13}C- and ^{18}O-labelled eicosanoids emerge from GC columns practically at the same time as their unlabelled analogs while derivatized ^{2}H-labelled eicosanoids emerge a few seconds earlier due to the lower polarity of ^{2}H compared with ^{1}H. Identical retention times on GC columns is not a drawback; moreover, this may facilitate the identification of a certain eicosanoid in some difficult analyses.

3.2.4. Solid-phase extraction and immunoaffinity column extraction

With minor exceptions, the first step of analytical methods in eicosanoids analysis is their extraction from a biological sample. In principle, two extraction techniques have been reported for the extraction of eicosanoids from biological samples ([86] see also Section 4]. These are solvent extraction [30,152] and SPE based on distinct physical or chemical principles [21,30,61,102,139,153–156]. A special case of SPE represents the use of immunoaffinity columns [63,120,157–160]. This technique is based on the use of a chemically immobilized antibody against the eicosanoid to be extracted. Unfortunately, immunoaffinity columns are not generally commercially available, so that this efficient technique is not accessible to every analyst interested in eicosanoids analysis. Today, solvent extraction of eicosanoids has been largely substituted by SPE. The major advantages of SPE over solvent extraction are: wider applicability, better efficiency, greater rapidity, and easier practicability. During the last fifteen years, SPE has been developed to the most efficient and indispensable extraction technique in GC–MS and other analytical techniques for isolation of arachidonic metabolites from biological samples, their purification and enrichment. Usually, extraction of eicosanoids from 1 to 5 ml of acidified biological material is carried out on reversed-phase cartridges consisting of octadecylsilica (C_{18}). Other reversed-phase materials such as phenylboronic acid (PBA) [155] and amino (NH_2) normal-phase [61] have been shown to be highly selective for some eicosanoids. Extraction with these materials yields highly purified extracts and considerably simplifies and shortens analysis of some eicosanoids [61,155]. Recently, SPE of LTE_4 from human urine for quantitation by EIA has been reported for the first time by using empore C_{18} disk cartridges [77]. The utility of such disks for the measurement of LTE_4 and other eicosanoids by GC–MS has not been reported so far.

3.2.5. Purification and separation by SPE, HPLC and TLC

Urine and plasma extracts from solvent extraction or SPE containing native or partially derivatized eicosanoids are subjected to further chromatographic analysis by means of SPE, HPLC and/or TLC for the purpose of purification and separation. Table 1 summarizes the most relevant purification and separation methods which have been used in GC–MS methods for the quantitation of eicosanoids in human urine and plasma. SPE has been used either for removal of derivatizing agent excess and structurally unrelated compounds or for separation of a particular eicosanoid from other related eicosanoids utilizing specific sorbents such as PBA [155,171,172]. Use of SPE for purification and isolation may considerably improve the signal-to-noise ratio of GC–MS detection and highly increase selectivity for eicosanoids

Table 1
Purification and separation methods for GC–MS analysis of eicosanoids in human urine and plasma based on solid-phase extraction (SPE), high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC)

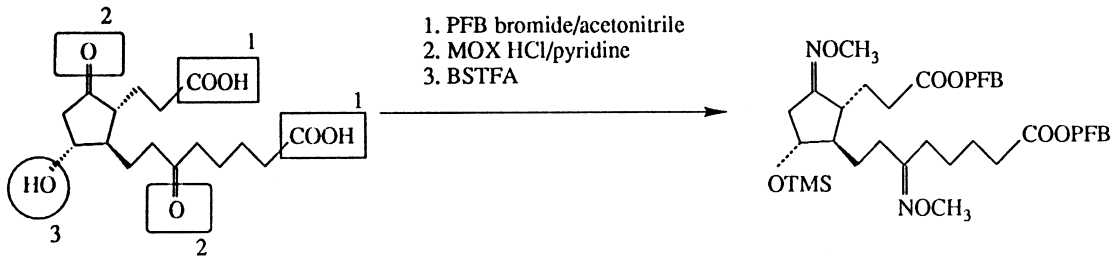
Method	References
SPE	
SiO_2	[88,95,114,134,135,139,142,161–170]
C_{18}	[135,139,164,165,167,168]
Amino	[119]
PBA	[155,171,172]
Immunoaffinity	[120,157–160]
HPLC	
Straight-phase	[95,102,161–163,165,173–176]
Reversed-phase	[69,74,95,122,131,132,162,166,167,169,177,178]
TLC	
Underivatized eicosanoids	[88,91,110,135,154,176,179–183]
Derivatized eicosanoids	[88,91,110–112,121–123,135,137,142,150, 155,168,172,173,179–181,184]

such as TxB_2 and 2,3-dinor-TxB_2 [155,171]. With the exception of these special cases, SPE does not bring on satisfactory purification so that additional chromatographic methods of greater separation efficiency such as HPLC and TLC are required. HPLC, both straight- and reversed-phase, and TLC have been used for the separation of native as well as derivatized eicosanoids. Separation of pentafluorobenzyl (PFB) esters of eicosanoids by HPLC or TLC is more advantageous in terms of rapidity and simplicity over systems separating the free acids because extraction of PFB esters from the respective fractions takes place more rapidly and efficiently without the need of acidification. While leukotrienes are most frequently separated by HPLC, prostanoids are preferably purified by TLC. HPLC and TLC do not only eliminate derivatizing reagent excess, they moreover separate eicosanoids from each other and from their isomers. These purification methods yield fractions which usually contain the desired eicosanoid alone in satisfactory purity so that subsequent GC–MS analysis can be performed. Also, simultaneous analysis of two or more eicosanoids present in separate HPLC fractions or TLC zones has been reported to be possible by GC–tandem MS [112,113]. For quantitation of many eicosanoids by GC–MS, it has been reported that both HPLC and TLC are necessary for satisfactory purification and separation. Renunciation of any HPLC or TLC for GC–MS or GC–tandem MS of eicosanoids is rare but possible for some eicosanoids [114,157,158,185]. Both HPLC and TLC are suitable for routine analysis of eicosanoids as both techniques can be automated to a high degree. In contrast to urinary LTE_4, for which a fully automated HPLC–RIA method has been developed [59], fully automated analysis of eicosanoids by GC–MS is obviously not possible.

3.2.6. Derivatization

Derivatization is a chemical reaction of an analyte with an appropriate derivatizing agent to yield a derivative of the analyte with improved physicochemical properties. In GC, derivatization is performed mainly to increase volatility and thermal stability of an analyte. Derivatization in quantitative GC–MS additionally aims to obtain derivatives with improved MS properties, for example in order to achieve maximum sensitivity due to weak fragmentation. Eicosanoids have many functionalities in their molecules so that several derivatizations have to be performed prior to GC–MS analysis. The most frequently used derivatization reactions in eicosanoid analysis include: (1) esterification of the carboxy group; (2) methoximation of the oxo functionality; and (3) etherification of the hydroxy group. Scheme 2 illustrates these derivatization reactions for PGE-M as an example of a prostanoid having three different functionalities.

In the past, eicosanoids have been frequently



Scheme 2. Chemical reactions, i.e. derivatizations, of the major urinary metabolite of E prostaglandins, PGE-M, to yield the PFB-MO-TMS derivative for GC–MS analysis in the NICI mode. 1, Esterification; 2, methoximation; 3, etherification; PFB, pentafluorobenzyl; MOX, methoxyamine; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide. For simplicity only one MO isomer per oxo group is shown.

converted to their methyl esters using diazomethane in ethereal solution for quantitation by GC–EI-MS. Preparation of methyl esters is still used for some dicarboxylic prostanoids [109,183]. Currently, eicosanoids are almost exclusively quantitated by GC–NICI-MS. For this purpose they are derivatized by PFB bromide to yield the strong electron-capturing PFB esters. It has been shown that considerable improvement of sensitivity can be achieved by using pentalfuorophenyldiazoalkanes [186]. However, these reagents could not replace PFB bromide as the esterifying agent. Esterification with PFB bromide is usually carried out in water-free acetonitrile using a secondary amine such as *N,N*-diisopropylethylamine (DIPEA) as a catalyst. Methoximation of oxo groups is frequently performed by using a saturated methoxyamine (MOX) hydrochloride solution in pyridine or in buffered urine. As a rule, this reaction yields two methoxime-isomers for an oxo group, i.e. the syn- and anti-methoxime derivatives, which may have different chromatographic and mass spectrometric properties [150,187]. Usually, etherification of hydroxy groups is carried out with pure *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) yielding trimethylsilyl (TMS) ethers.

For eicosanoids having 1,2- or 1,3-diols such as for $PGF_{2\alpha}$, esterification of these hydroxy groups by *n*-butylboronic acid to yield cyclic boronate derivatives is an alternate derivatization reaction [123,188,189]. Since boronic esters are less volatile than for instance TMS ethers, preparation of boronic esters is rather of qualitative than of quantitative value [123]. Thus, this derivatization reaction can be utilized to distinguish between α- and β-configurated hydroxy groups in the cyclopentane ring of eicosanoids as has been shown for PGF_2 isomers [188,189].

When eicosanoids have to be quantitated by GC–NICI–MS, some points concerning derivatization should be taken into consideration. Some eicosanoids such as 2,3-dinor-6-oxo-$PGF_{1\alpha}$ [154], PGF-M [137], 11-dehydro-TxB_2 [190], 5-HETE [22,191], and 20-hydroxy-LTB_4 [192] may occur in two different forms, for example as free acid and as lactone, depending on the respective pH of the biological sample or of an extract. A further point concerns the sequence of the performance of the various derivatization steps. This may depend on the eicosanoid or on the respective extraction procedures used. As a rule, esterification of the carboxy group precedes the silylation of the hydroxy group because silylating agents such as BSTFA readily convert the carboxy group to its TMS ester. Usually, silylation of the hydroxy group(s) is the last derivatization step. Also, derivatization does not only yield the desired derivative but it also does lead to many side-products that may deteriorate both chromatographic and mass spectrometric analysis. This often makes purification of reaction products necessary including elimination of excess of derivatizing agents such as PFB bromide. After completed derivatization, samples can usually be stored at 4°C in a few microlitres of pure BSTFA or in solutions of BSTFA in organic solvents such as hexane for many days or weeks without remarkable deterioration and loss of sensitivity.

Although there exists only a few different derivatization reactions in GC–MS analysis of eicosanoids, there have been reported in the literature many variants of these reactions with regard to solvent, excess of derivatizing agent, temperature

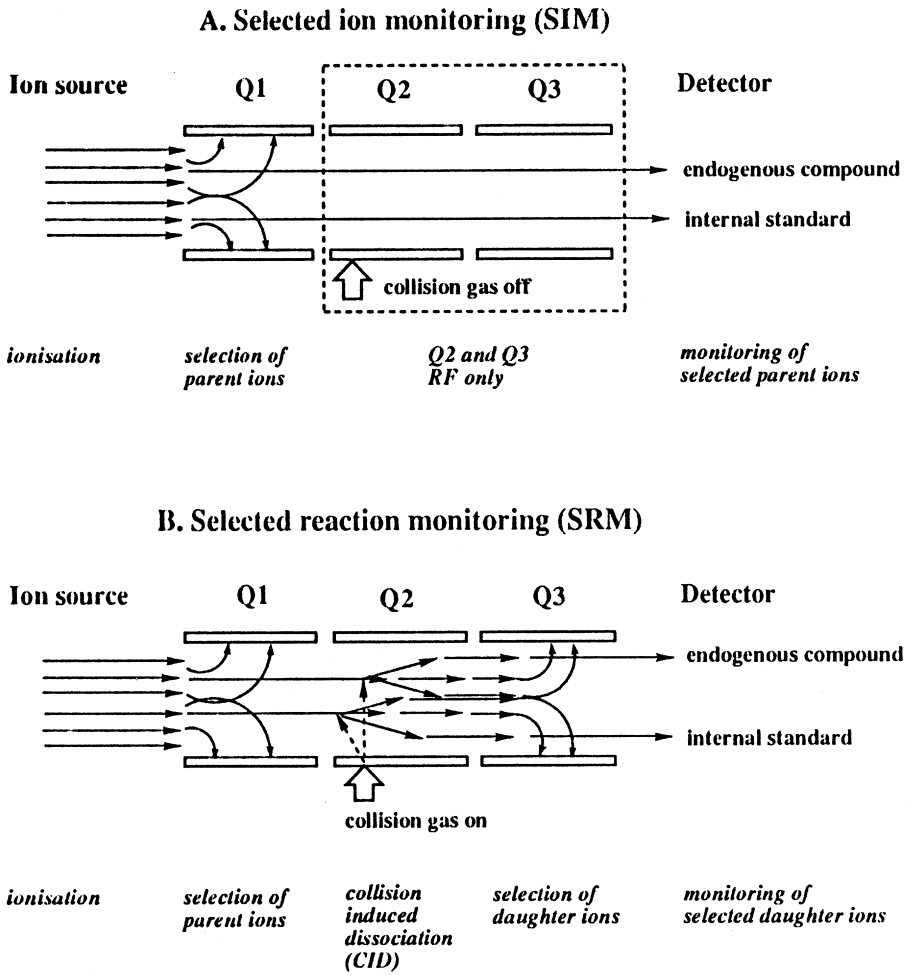
and duration of the reaction, and storage of the samples to be injected into the GC–MS apparatus. Derivatization procedures commonly used in eicosanoid analysis, in particular in GC–NICI-MS, have been described in detail [74,102,111,114] [119–123,131,133,135] [137,142,150,154,155,157–159, 164–166,168–171,173,174,176–178] [181,183,184, 188–197].

3.2.7. Principles of operation techniques in GC–MS and GC–tandem MS

Single-stage quadrupole (SSQ) and triple-stage quadrupole (TSQ) mass spectrometers coupled with gas chromatographs are the most convenient and frequently used analytical tools in quantitative analysis of eicosanoids in biological fluids. The quadrupole of SSQ instruments and the first and the third quadrupole of TSQ instruments are operated by combination of direct current (DC) voltages and radio frequency (RF). In TSQ instruments, the second quadrupole is operated in the RF-only mode and mainly serves as a high-pressure collision cell. Major advantages of quadrupole instruments over other mass spectrometers include operational simplicity, rapid scanning capabilities, unit mass resolution, small size, relative low cost and satisfactory mass range for most eicosanoids derivatives. On the other hand, lack in high resolution is the main drawback of quadrupole mass spectrometers. For a more detailed description of instrumentation and principles of operation techniques including ionization techniques with SSQ and TSQ mass spectrometers and other mass spectrometers refer to the literature [54,99,198–203].

The principles of the two most important operations with SSQ and TSQ mass spectrometers for quantitative measurements are schematically illustrated in Scheme 3. Using SSQ or TSQ instruments operating in the single stage, mass spectra can be generated from an analyte ionized in the ion source by scanning the quadrupole over an expected mass-to-charge (m/z)-range (not shown). NICI of PFB-TMS and PFB-MO-TMS derivatives of eicosanoids using methane or isobutane as the reactant gases produces mass spectra poor in mass fragments. An example for a typical GC–NICI-MS spectrum is shown in Fig. 1A for the PFB-MO-TMS derivative of PGE-M. The most intense ion in GC–NICI-MS spectra of PFB esters of eicosanoids is a stabilized carboxylate anion $[M-PFB]^-$ which is formed by cleavage of a highly stabilized PFB radical that follows capture of an electron by the strong electron-capturing PFB moiety of the PFB ester. Further mass fragments of substantially lower intensity are formed due to consecutive loss of TMSOH group(s). Unlike GC–EI-MS, GC–NICI-MS does not lead to cleavage of C-C bonds. This method of scanning has proved to be useful in identifying LTE_4 and 20-carboxy-LTE_4 in urine of diseased humans requiring only pg amounts [66,69,204–206].

$[M-PFB]^-$ ions correspond to the molecular mass M of the derivatized eicosanoids minus the PFB moiety. Because of the high intensity and specificity of these ions, monitoring of $[M-PFB]^-$ mass fragments of PFB-(MO)-TMS derivatives of eicosanoids by GC–NICI-MS provides the most sensitive and specific method in quantitative eicosanoid analysis in the so-called selected ion-monitoring (SIM) or multiple ion detection (MID) mode (Scheme 3A). With SSQ and TSQ instruments, this is accomplished simply by alternate setting of the first quadrupole to pass the $[M-PFB]^-$ ions of the endogenous eicosanoid and of its stable isotope-labelled analog and by alternate detection of these ions. Despite selection of specific $[M-PFB]^-$ ions in the SIM mode, this technique lacks in selectivity so that eicosanoids cannot be specifically quantitated in human urine and plasma except when extensive purification has been performed. PFB-MO-TMS derivatives of various eicosanoids such as 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 2,3-dinor-TxB_2 may be ionized to $[M-PFB]^-$ ions with identical m/z values. Further example for unselectivity are numerous COX-dependent and COX-independent F_2 prostaglandins ($[M-PFB]^-=569$). These eicosanoids are present in human urine at concentrations several orders of magnitude higher than that of LTB_4 ($[M-PFB]^-=479$), so that their less intense ions of PGF_2 such as $[M-PFB-TMSOH]^-=479$ could interfere with the quantitation of LTB_4 when GC coelution takes place. Also, a variety of unknown compounds that coelute with PFB-(MO)-TMS derivatives of eicosanoids and produce ions identical to their $[M-PFB]^-$ ions may interfere in quantitative analysis by SIM. Complete chromatographic separation of the PFB-MO-TMS derivatives prior to SIM is therefore an absolute



Scheme 3. Schematic drawing of operation techniques using single-stage and triple-stage quadrupole instruments. (A) Quantitative analysis by selected-ion monitoring (SIM) of two ions specific for an endogenous analyte and for its stable isotope-labeled analog. (B) Quantitative analysis by selected-reaction monitoring (SRM) of two specific daughter ions generated by CID of the corresponding parent ions. One pair of ions is chosen for the endogenous compound, the corresponding second pair of ions is chosen for the internal standard. Q, quadrupole.

requirement for selective quantitation. Unselectivity in quantitative analysis of eicosanoids in human urine and plasma by SIM can be overcome by the use of extensive separation and purification procedures (see Section 4).

A less laborious and less time-consuming elimination of problems associated with SIM represents the GC–tandem MS as can be performed in TSQ instruments. In GC–tandem MS a $[M-PFB]^-$ ion, the so-called parent ion $[P]^-$, selected by the first quadrupole is subjected to collisionally activated dissociation (CAD) or collision-induced dissociation (CID) in the second quadrupole by a collision gas such as argon. In this process, the $[M-PFB]^-$ ion is fragmented to the so-called daughter ions which can be subsequently analysed by the third quadrupole. Scanning of the third quadrupole over a m/z-range including that of the parent ion produces the daughter ion mass spectrum (not shown). Fragmentation pattern and relative intensity of daughter ions mainly depend on argon pressure in the collision chamber and on collision energy. Fig. 1B shows the NICI daughter ion mass spectrum of the PFB-MO-TMS derivative of PGE-M. Daughter ion mass spectra of

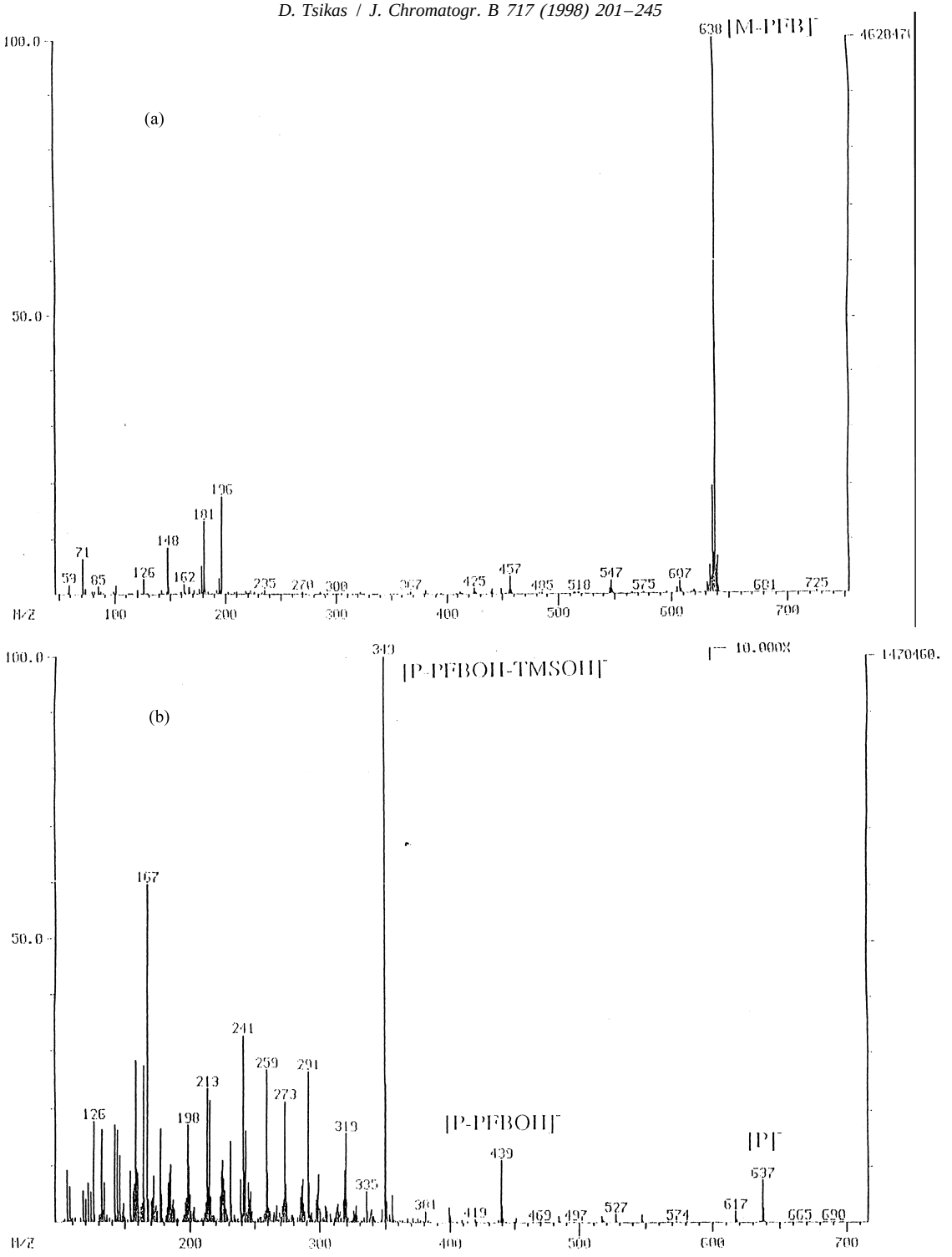


Fig. 1. (A) GC–NICI-MS spectrum of the major peak of the diPFB-diMO-TMS derivative of PGE-M. The most intense mass fragment at m/z 637.7 represents the carboxylate anion, e.g. $[M-PFB]^-$. (B) Daughter-ion mass spectrum generated from the diPFB-diMO-TMS derivative of PGE-M by CID of the parent ion $[M-PFB]^-$, $[P]^-$, at m/z 637.7.

PFB-(MO)-TMS derivatives of dicarboxylic eicosanoids are characterized by intense mass fragments which are formed from the parent ions $[M-PFB]^-$ by the loss of a PFBOH group from the second carboxylic group and consecutive loss of a TMSOH group. This kind of fragmentation was observed for instance for PGE-M (Fig. 1B) [131,150], PGF-M [150], 20-carboxy-LTB_4 [69,207], and ω-carboxylated LTE_4 metabolites [132]. Daughter-ion mass spectra show intense mass fragments including those from the loss of methoxy groups from the methoxime groups and carbon dioxide from the carboxylic group. Further cleavage of C-C bonds by CID in GC–NICI-MS–MS is a rare phenomenon and has been observed for LTE_4 and its carboxylated metabolites [129,132,151] and LTB_4 [113]. CID of the parent ions $[M-PFB]^-$ of PFB-TMS derivatives of catalytically hydrogenated LTE_4, LTB_4 and their metabolites yields highly characteristic daughter ions $[M-PFB-146]^-$. The mechanism of the CID of PFB-TMS derivatives of catalytically hydrogenated LTE_4 has not been fully understood. Since the formation of the daughter ion at m/z 253 $[M-PFB-146]^-$ has not been observed from the PFB-TMS derivatives of reduced 12-HETE and 15-HETE [132] the hydroxy group at the position five seems to be exclusively involved in the CID process. Further, CID of the PFB-TMS derivative of [1,1-$^{18}O_2$]HEA from catalytical hydrogenation of [1,1-$^{18}O_2$]LTE_4 yields also the same daughter ion, i.e. m/z 253, as that of LTE_4, suggesting loss of the carboxylic group [113]. Daughter ions are characteristic of the structure of the parent ion. This method of scanning has proved to be useful in identifying unequivocally LTE_4 and LTB_4 in pg amounts in urine of diseased humans [69,105].

In quantitative analysis by TSQ instruments in the so-called selected-reaction monitoring (SRM) or multiple-reaction monitoring (MRM) mode, the first quadrupole is set to pass alternately the parent ions $[M-PFB]^-$ of the endogenous eicosanoid and of its stable isotope-labelled analog while the third quadrupole is set to pass concomitantly the corresponding specific daughter ions formed in the collision chamber of the second quadrupole (Scheme 3B). Although commonly referred to as MS–MS, tandem mass spectrometric analysis is usually MS–CID–MS. Typical parent and corresponding daughter ions used for quantitative analysis of eicosanoids in human urine and plasma by SIM and/or SRM are summarized in Table 2.

GC–tandem MS highly increases selectivity of eicosanoid analysis and makes accurate analysis where simple GC–MS fails. Thus, in the case of GC coelution of compounds with identical $[M-PFB]^-$ as could occur for the PFB–MO–TMS derivatives of 6-oxo-$PGF_{1\alpha}$ and TxB_2, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 2,3-dinor-TxB_2 or for the PFB-TMS derivatives of reduced LTE_4 and HETEs, and for many other compounds, GC–tandem MS enables selective quantitative analysis as different characteristic daughter ions from identical parent ions $[M-PFB]^-$ can be obtained. Catalytical hydrogenation of some hydroxylated polyunsaturated eicosanoids such as LTB_4 [113] to saturated derivatives may considerably alter the daughter ion pattern. Fig. 2 shows daughter ion NICI mass spectra of the PFB-TMS derivatives of unsaturated (A) and saturated LTB_4 (B). CID of the parent ion $[M-PFB]^-$ at m/z 487 of the PFB-TMS derivative of saturated LTB_4 produces, probably by a mechanism similar to hydrogenated LTE_4, a daughter ion at m/z 341 ($[M-PFB-146]^-$) which is characteristic for dihydroxy-eicosanoic acids carrying one hydroxy group at position five. Hydrogenation of LTB_4 does not only enhance sensitivity due to increased thermal stability of the PFB-TMS derivative and specificity of GC–tandem MS but it also enables simultaneous quantitation of LTE_4 and LTB_4 in biological fluids [113].

Besides these scanning techniques, TSQ instruments enable parent-ion scan and neutral fragment-loss scan. The capability of the TSQ instrument to perform all these scanning techniques together with the high separation efficacy of capillary columns make the combination of TSQ with gas chromatographs the instrument of choice in quantitative and qualitative analysis of eicosanoids in biological fluids.

Provided that a sample to be injected into GC–MS instruments for purpose of quantitation by SIM or SRM contains several eicosanoids having identical or distinct parent ions and/or daughter ions and that no interferences are present in the sample, SSQ and TSQ instruments allow simultaneous quantitation of

Table 2
Parent and major daughter ions of PFB-(MO)-TMS derivatives of some unlabeled primary eicosanoids and their index metabolites generated in the NICI mode

Eicosanoid	Parent ion	Major daughter ion	
	m/z of $[M-PFB]^-$	m/z	References
Prostanoids			
6-oxo-$PGF_{1\alpha}$	614	312	[187]
6,15-dioxo-13,14-dihydro-$PGF_{1\alpha}$	571	481	[84]
2,3-dinor-6-oxo-$PGF_{1\alpha}$	586	240	[187]
TxB_2	614	268	[187]
2,3-dinor-TxB_2	586	240, 272	[187,169]
11-dehydro-TxB_2	511/615[a]	243/345[a]	[142], [183][a]
PGE_1	526	270	[208]
13,14-Dihydro-15-oxo-PGE_1	483	331	[130]
PGE_2	524	268	[187]
PGE-M	637	439, 349	[131,150]
PGD_2	524	268	[187]
PGD-M	514[b]		
$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, PGF_2	569/491[c]	299/317[c]	[123], [187][c]
PGF-M	682	484	[150]
Leukotrienes			
LTB_4	479/487[d]/563[e]	299/341/299[f]	[175,113], [207][f]
20-OH-LTB_4	693[e]		
20-COOH-LTB_4	689/773[e]	491	[69]
LTC_4, LTD_4, LTE_4	399	253	[129]
20-COOH-LTE_4	609	411	[132]
18-COOH-LTE_4	581	383	[132]
16-COOH-LTE_4	557	355	[132]
HETEs			
5-HETE	399[g]	253[g]	[132][g]
12-HETE	441[h]		
15-HETE	441[h]		
20-HETE	433[i]		

[a] PFB-Me-TMS derivative; [b] PFB-Me-MO-TMS derivative; [c] PFB-*n*-butylboronate-TMS derivative; [d] saturated LTB_4; [e] PFB-*t*-butyldimethylsilyl derivative; [f] m/z 299 is the daughter ion of m/z 431, i.e. $[M-PFB-2x\textit{t}\text{-butyldimethylsilanol}]^-$; [g] saturated 5-HETE; [h] PFB-*t*-butyldimethylsilyl derivative of saturated HETEs; [i] PFB-*t*-butyldimethylsilyl derivative of unsaturated 20-HETE.

several eicosanoids extracted from urine or plasma. For eicosanoids with satisfactorily different retention times on capillary columns, quantitation can be performed at maximum sensitivity for each eicosanoid. This has been demonstrated for prostaglandins [112] and leukotrienes [113] by combining separate fractions from TLC zones or HPLC fractions containing the respective eicosanoids.

Selection of specific parent ions, their CID and additional selection of specific daughter ions by means of TSQ instruments enormously increases the specificity of quantitative eicosanoid analysis in complex biological fluids. The detection limit of quantitative analysis of PFB-(MO)-TMS derivatives of eicosanoids by SRM in the NICI mode with modern TSQ instruments is below 1 pg injected on column. It is worth mentioning that only a very small part, e.g 1/10th to 1/50th, of the sample available is injected into the instruments. This high sensitivity enables accurate quantitation of most eicosanoids in the lower pg/ml range in only 1-ml aliquots of urine or in 5- to 10-ml aliquots of human plasma.

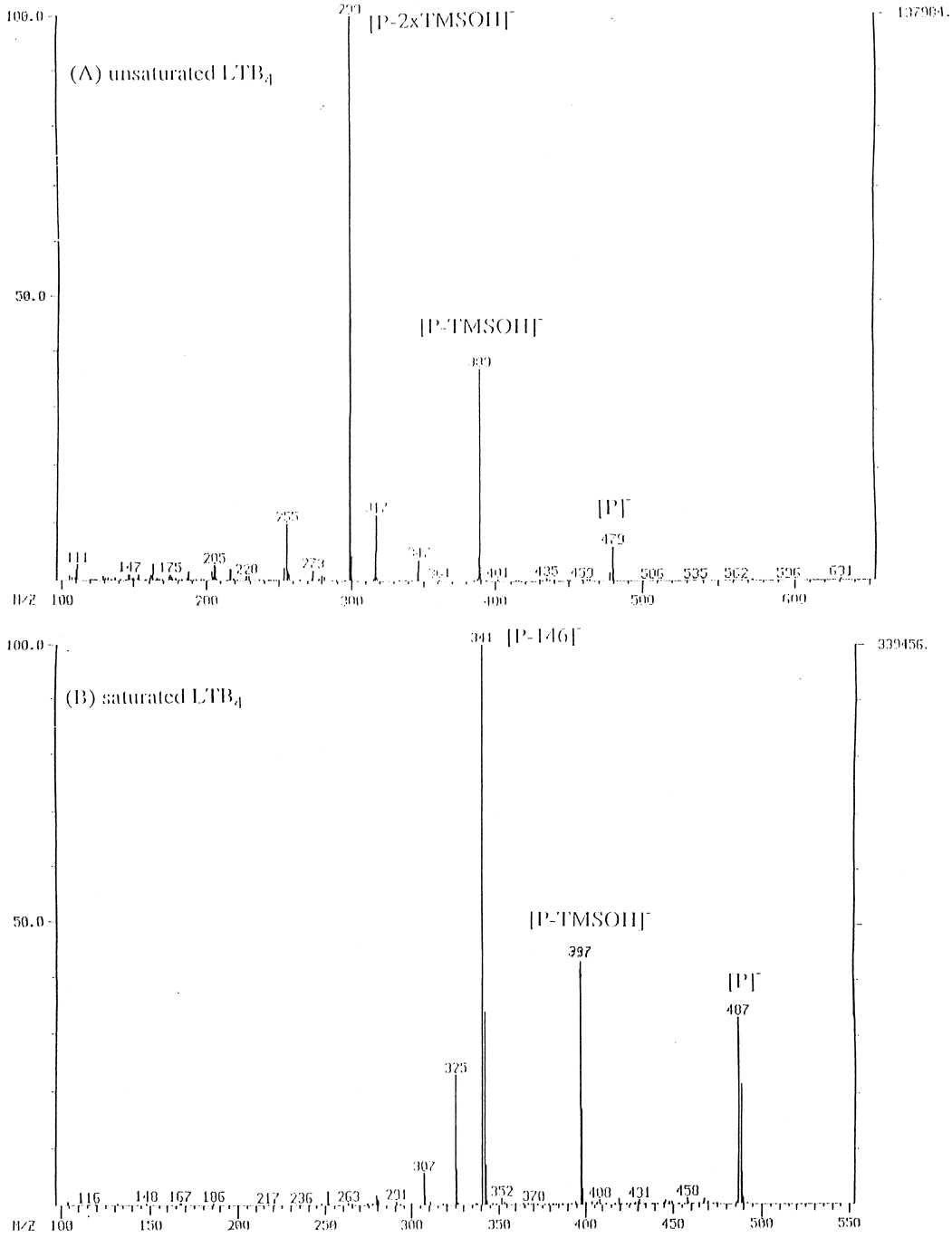


Fig. 2. Daughter-ion mass spectra of the PFB-TMS derivatives of (A) unsaturated and (B) saturated LTB_4. The parent ions $[M-PFB]^-$, $[P]^-$, at m/z 479 and 487, respectively, were subjected to CID.

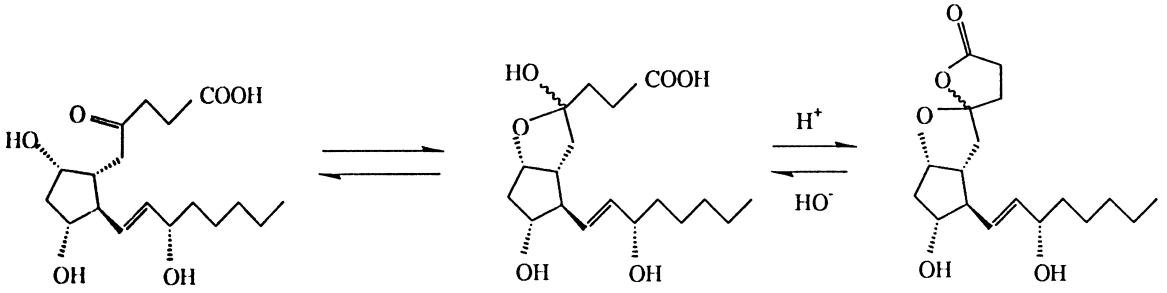
4. Methodological applications

4.1. Prostanoids

4.1.1. Prostaglandins

4.1.1.1. *Prostacyclin and index metabolites*. In 1976, Moncada et al. discovered prostacyclin (PGI_2) in the vascular endothelium [209]. At physiological pH, prostacyclin is highly unstable and spontaneously hydrolyses to the stable 6-oxo-$PGF_{1\alpha}$ [210]. Rosenkranz et al. have found by GC–EI-MS that the major urinary metabolite of both prostacyclin and 6-oxo-$PGF_{1\alpha}$ infused into healthy volunteers is 2,3-dinor-6-oxo-$PGF_{1\alpha}$ [211]. Besides this metabolite, 15-oxo-13,14-dihydro-2,3-dinor-6-oxo-$PGF_{1\alpha}$ has also been found to be an abundant urinary metabolite of prostacyclin and 6-oxo-$PGF_{1\alpha}$. 6-oxo-$PGF_{1\alpha}$ and 6,15-dioxo-13,14-dihydro-$PGF_{1\alpha}$ were found to be minor urinary excretory products of administered prostacyclin and 6-oxo-$PGF_{1\alpha}$. One year later, FitzGerald et al. have estimated the rate of prostacyclin secretion into the circulation of normal man by measuring 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 15-oxo-13,14-dihydro-2,3-dinor-6-oxo-$PGF_{1\alpha}$ in urine after infusion of prostacyclin by GC–EI-MS in the SIM mode [212]. In the same year, Falardeau et al. have reported this GC–EI-MS method in detail and the first urinary 2,3-dinor-6-oxo-$PGF_{1\alpha}$ levels from endogenous prostacyclin in normal human volunteers [154]. In their assay Falardeau et al. have utilized the ability of both dinor metabolites of 6-oxo-$PGF_{1\alpha}$ to exist in several forms in dependence of the pH [154]. Scheme 4 illustrates this context for 2,3-dinor-6-oxo-$PGF_{1\alpha}$. The lactone forms of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 15-oxo-13,14-dihydro-2,3-dinor-6-oxo-$PGF_{1\alpha}$ are spontaneously formed upon acidification (pH 5 or below), are sufficiently stable against hydrolysis during extraction of the organic solvent with mild aqueous base but they readily and rapidly hydrolyse to the free acids by using a mixture of water–pyridine–triethylamine. Falardeau et al. have reported that for quantification of low levels of the dinor metabolites in urine, for example from subjects receiving prostacyclin-inhibiting drugs like aspirin, an additional step of purification by TLC was necessary [154]. Imprecision and accuracy of the method for urinary 2,3-dinor-6-oxo-$PGF_{1\alpha}$ have been reported as 2% and 95%, respectively. From the two dinor metabolites of prostacyclin, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ was found to be the major metabolite of prostacyclin in the urine of healthy humans and became generally accepted as the index parameter of prostacyclin.

On the basis of the approach developed by Falardeau et al. [154], several modifications of the original, accurate but time-consuming and cumbersome method have been performed [111,164,184,185]. Daniel et al. [111] have simplified the GC–MS assay for urinary 2,3-dinor-6-oxo-$PGF_{1\alpha}$ by replacement of many of the differential extraction steps by reversed-phase column extraction and by simultaneous opening of the lactone and methoximation as had been previously shown for PGD-M, the major urinary metabolite of PGD_2 [135]. These changes have been reported to make two time-consuming TLC separations and opening of the lactone unnecessary so that approximately fifteen 3-ml urine samples can be processed and analysed by GC–NICI-MS in a 7-h period [111]. Impecision, accuracy and lower detection limit of this assay have been reported as 5%, 98% and 15 pg/mg creatinine,



Scheme 4. Free acid, hemiketal and lactone forms of 2,3-dinor-6-oxo-$PGF_{1\alpha}$.

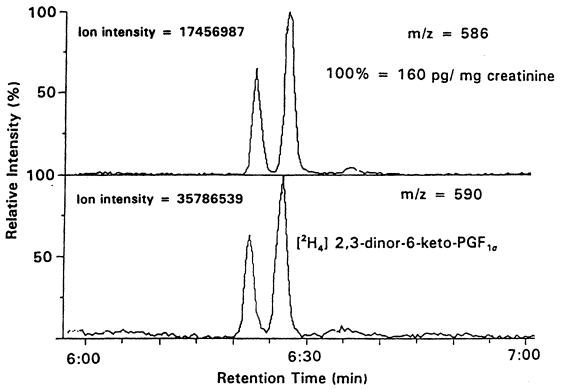


Fig. 3. GC–NICI-MS analysis of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in human urine from a normal healthy volunteer by a modification [111] of the original method first reported by Falardeau et al. [154]. SIM of m/z 586 for endogenous 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and m/z 590 for tetradeutero-2,3-dinor-6-oxo-$PGF_{1\alpha}$. From Ref. [111] with permission.

respectively. A chromatogram from the GC–NICI-MS analysis of urinary 2,3-dinor-6-oxo-$PGF_{1\alpha}$ by the method of Daniel et al. is shown in Fig. 3. Using a similar procedure to that reported by Daniel et al., we found that this method can be substantially shortened by omiting the TLC step when quantification by GC–NICI-MS–MS is performed [185]. A typical chromatogram obtained by this method is shown in Fig. 4. Imprecision, accuracy and lower detection limit of our assay were determined as 4%, 95% and 25 pg/ 5 ml, respectively. Ferretti and Flanagan [184] have also modified the original method of Falardeau et al. [154] and remarkably shortened the analysis of urinary 2,3-dinor-6-oxo-$PGF_{1\alpha}$. However, although analysis was performed by GC–NICI-MS–MS, the use of a TLC purification step in this method appeared to be necessary. Ferretti and Flanagan reported that imprecision and accuracy

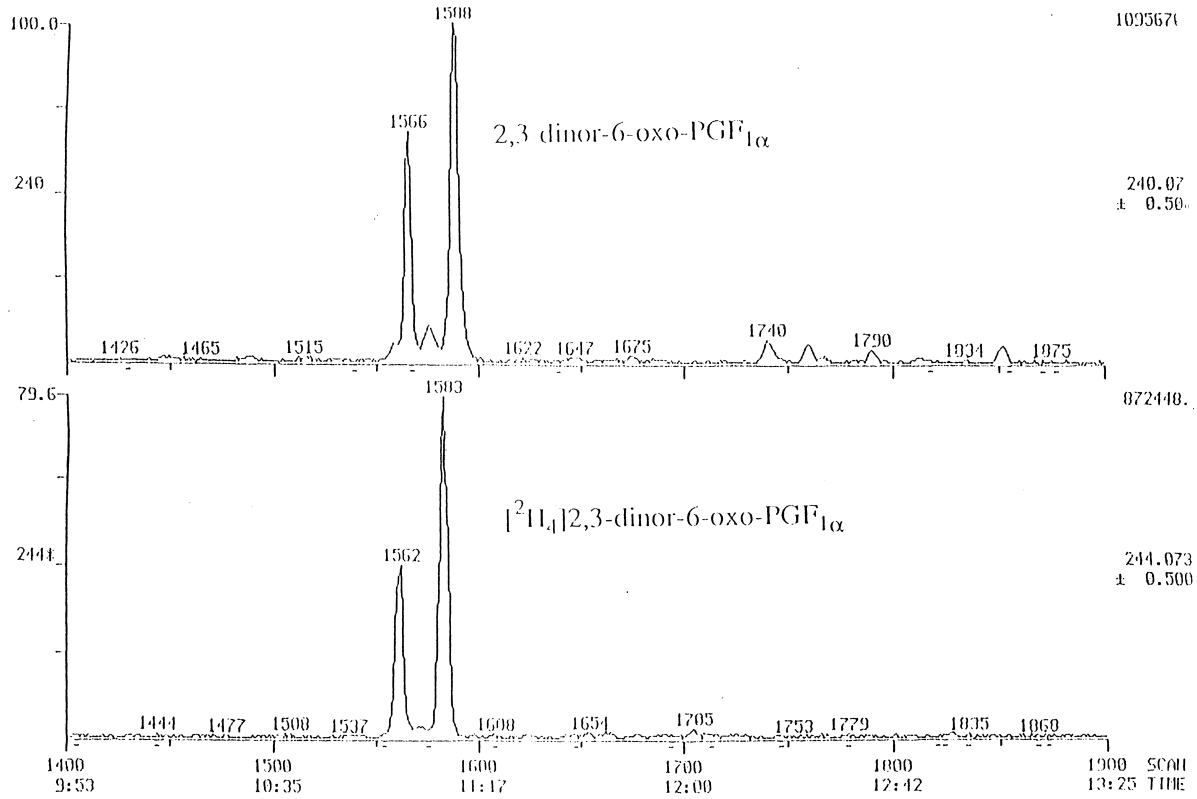


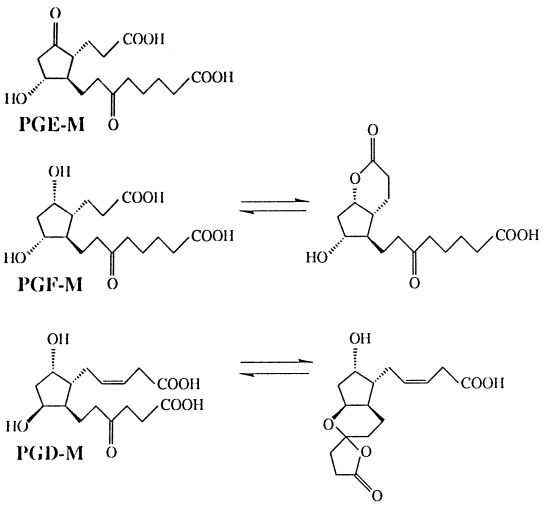
Fig. 4. GC–NICI-MS–MS analysis of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in human urine from a normal healthy volunteer by using a similar procedure to that reported by Daniel et al. [111] but without the TLC purification step [185]. SRM of m/z 240 for endogenous 2,3-dinor-6-oxo-$PGF_{1\alpha}$ (upper trace) and m/z 244 for tetradeutero-2,3-dinor-6-oxo-$PGF_{1\alpha}$ (lower trace).

of their assay were 5% and 85%, respectively. Mizugaki et al. have shown that another possibility to eliminate substances from human urine samples interfering with 2,3-dinor-6-oxo-$PGF_{1\alpha}$ can be achieved by high-resolution SIM GC–MS in combination with a series of SPE procedures [170]. Imprecision and accuracy of this method were reported to be 5% and 107%, respectively.

Besides these specific methods for 2,3-dinor-6-oxo-$PGF_{1\alpha}$, there have been reported numerous GC–MS methods in which 2,3-dinor-6-oxo-$PGF_{1\alpha}$ was one urinary analyte among many other prostanoids including 6-oxo-$PGF_{1\alpha}$, TxB_2, PGE_2, $PGF_{2\alpha}$ and their index metabolites [112,131,159,172]. The development of such methods resulted from the desire to simultaneously determine renal as well as systemic prostanoid synthesis. Chiabrando et al. have reported a rapid and simple GC–NICI-MS method based on mixed-bed immunoaffinity extraction employing different polyclonal antibodies against 6-oxo-$PGF_{1\alpha}$ and TxB_2 showing the superior specificity of immunoaffinity extraction over SPE or solvent extraction [159]. This method enabled simultaneous paired-determination of 6-oxo-$PGF_{1\alpha}$ and TxB_2 and their major urinary metabolites. Imprecision and accuracy of the method for 2,3-dinor-6-oxo-$PGF_{1\alpha}$ were reported as 2.5% and 93%, respectively. Weber et al. reported a GC–NICI-MS method for urinary prostanoids including 2,3-dinor-6-oxo-$PGF_{1\alpha}$ [172]. They methoximated prostanoids directly in human urine and subjected them to SPE on PBA cartridges, thus separating TxB_2 and 2,3-dinor-TxB_2 from 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and other prostanoids. After repeat SPE of the two fractions, the methoxime derivatives were subjected to TLC prior to separate analysis by SIM. Imprecision and accuracy of this assay for 2,3-dinor-6-oxo-$PGF_{1\alpha}$ were reported to be 7% and 90%, respectively. Fauler et al. have used reversed-phase HPLC to separate PFB-MO-derivatives of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and other urinary prostanoids prior to quantification by GC–NICI-MS–MS [131]. Schweer et al. have separated PFB-MO derivatives of 2,3-dinor 6-oxo-$PGF_{1\alpha}$ and other urinary prostanoids by TLC prior to quantification by GC–NICI-MS–MS [112]. By this assay 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 6-oxo-$PGF_{1\alpha}$ were determined simultaneously. The usefulness of this method for the quantification of prostanoids by GC–NICI-MS has not been reported by this group. Further GC–MS methods for the quantitative determination of 6-oxo-$PGF_{1\alpha}$ alone, together with 2,3-dinor-6-oxo-$PGF_{1\alpha}$, or with other prostanoids in human urine and plasma have been reported for the past fifteen years [160–162,173,174,177,196,213]. Unlike 2,3-dinor-6-oxo-$PGF_{1\alpha}$, the inability of 6-oxo-$PGF_{1\alpha}$ to form a lactone makes GC–MS methods unequally time-consuming except for methods using immunoaffinity extraction.

Regarding the measurement of circulating prostanoids, But and Buchanan have shown that the sampling technique influences the basal level of 6-oxo-$PGF_{1\alpha}$ in plasma [101]. Instead of 6-oxo-$PGF_{1\alpha}$, Frölich et al. have suggested measuring 6,15-dioxo-13,14-dihydro-$PGF_{1\alpha}$, which is a circulating metabolite of prostacyclin in man [214,215], that cannot be formed in vitro. This prostacyclin metabolite has been determined in human plasma by GC–NICI-MS–MS following HPLC separation [84]. GC–NICI-MS was found by this group to be unsatisfactory due to many interferences.

4.1.1.2. *PGE_2, PGD_2, PGF_2 and index metabolites*. The first two classical prostaglandins, i.e. PGE and PGF, have been isolated and structurally elucidated by Bergström and its group [216,217]. The biosynthesis of PGE_2 from arachidonic acid was independently shown by the groups of Dorp [218] and Bergström [219]. PGD_2 is the major COX product produced by mast cells [220]. Hamberg and Samuelsson have investigated the metabolism of PGE_1 and PGE_2 in man and found by GC–EI-MS that the major urinary metabolite of intravenously administered PGE_1 and PGE_2 was 7α-hydroxy-5,11-dioxo-tetranor-prostane-1,16-dioic acid (PGE-M) [118]. In this work they also described a GC–EI-MS method for the quantitative determination of PGE-M in human urine and reported first levels for urinary excretion of PGE-M. The major urinary metabolite of $PGF_{2\alpha}$ in man was identified as 5a,7α-dihydroxy-11-oxo-tetranor-prosta-1,16-dioic acid (PGF-M) by Granström and Samuelsson [221]. Liston and Roberts have investigated the metabolic fate of radiolabelled PGD_2 in a normal human and identified by GC–EI-MS its major urinary metabolite as 9a,11β-dihydroxy-15-oxo-tetranor-prost-5-ene-1,20-



Scheme 5. Chemical structures of PGE-M, PGF-M and PGD-M, the major urinary metabolites of PGE_2, $PGF_{2\alpha}$ and PGD_2, respectively.

dioic acid (PGD-M) indicating predominant conversion of PGD_2 to PGF-ring metabolites in humans [188]. Scheme 5 shows the chemical structures of PGE-M, PGF-M and PGD-M and their pH-dependent equilibria. Also, Liston and Roberts have shown that PGD_2 is enzymatically transformed in vivo in humans to the biologically active 9a,11β-PGF_2 [189]. These studies showed potential analytical problems associated with quantification of $PGF_{2\alpha}$ and its metabolites in biological fluids, especially under conditions of elevated production of isomeric PGF-ring metabolites arising from PGD_2 metabolism such as asthma and anaphylaxis, because $PGF_{2\alpha}$ and its metabolites may not always be completely separated from 9a,11β-PGF_2 and its metabolites by HPLC, TLC and GC. In such situations, the use of *n*-butylboronic acid, which reacts only with the 9a,11α-dihydroxy groups of the cyclopentane ring of $PGF_{2\alpha}$ and its metabolites, may take remedial measures [188,189].

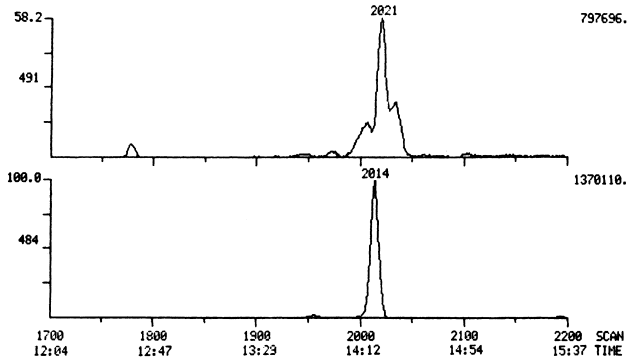
Hamberg introduced the use of the [$^2H_3$]methoxime-dimethyl derivative of PGE-M as an internal standard for the GC–EI-MS determination of PGE-M in human urine [118,222]. Gill et al. [223] and Seyberth et al. [138] have used this PGE-M derivative as an internal standard for GC–EI-MS quantification of urinary PGE-M and showed the method's applicability to clinical studies. Ferretti et al. have tried to overcome the lack of a suitable stable isotope-labelled PGE-M and PGF-M by using homologous internal standards of PGE-M [139] and PGF-M [195]. Imprecision and accuracy of these GC–EI-MS methods were reported to be of the order of 5% and 100%, respectively. The lower limit of detection for PGF-M was reported as 2 ng/ml when a 20-ml aliquot of urine was analysed. [195]. The first use of a ^2H-labelled PGE-M as an internal standard was described by Erlenmaier et al. [224] and Walker et al. [225] applying published analytical methods [138,222]. Erlenmaier et al. have reported disturbance of quantitative analysis of PGE-M by unresolved isomers and a falling base-line despite the use of intensive purification and isolation procedures [224]. They also reported that the introduction of a capillary column instead of packed GC columns resulted in more accurate analysis. Walker et al. have reported a detection limit of 0.5 ng/ml urine with a 20-ml urine sample in their GC–EI-MS assay [225].

Hamberg reported the first GC–EI-MS method for the quantitative analysis of PGF-M in human urine using the methoxime derivative of a tritiated/tetradeuterated PGF-M standard [136]. Because of the loss of deuterium, the standard had to be prepared and used as its methoxime derivative. The method has been reported to be precise (2% to 4%) and accurate (97%) but to require large volumes of urine (5% of a 24-h portion) and extensive purification by reversed-phase partition chromatography and TLC. Hamberg found that PGF-M remained almost unchanged when incubated in human urine for 5 h at 37°C [136]. Brash et al. [137] have simplified and refined the GC–MS method of Hamberg [136]. They synthesized a tetradeuterated PGF-M internal standard and used it as methyl ester [137]. Under alkaline conditions, both internal standard and endogenous PGF-M were quantitatively converted to the same chemical species so that the tetradeuterated PGF-M standard functioned as a real internal standard. Precision, accuracy and lower limit of detection of this GC–EI-MS assay were reported to be 2%, 100% and 1 ng/ml, respectively. Nevertheless, the method of Brash et al. was still very time-consuming as only 20 samples could be processed by one person per week.

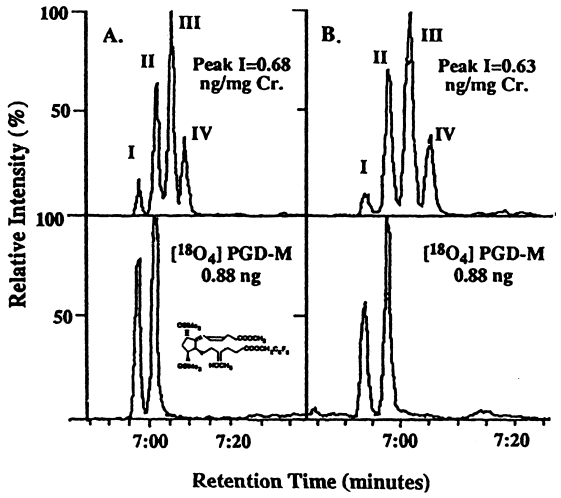
Considerable improvement in terms of sensitivity and simplicity in quantitative analysis of PGE-M, PGF-M and PGD-M in human urine was achieved by NICI in combination with the use of ^{2}H- and ^{18}O-labelled analogs of these prostaglandins [109,112,131,135–137,150]. Chromatograms from the analysis of PGF-M (A), PGE-M (B), and PGD-M (C) in human urine are shown in Fig. 5. Schweer et al. reported determination of PGE-M and PGF-M in 1-ml aliquots of human urine by GC–NICI-MS–MS using their ^{2}H-labelled analogs [150]. The method involved methoximation in urine, purification of the PFB–MO derivatives by TLC followed by quantification with GC–NICI-MS–MS. Unlike PGE-M, PGF-M occurs in human urine as a δ-lactone which has to be opened prior to methoximation so that PGE-M and PGF-M had to be determined separately in two different urine samples (Fig. 5a) [150]. Schweer et al. have shown by direct comparison of [$^{2}H_3$]methoxime derivative of PGE-M and [$^{2}H_7$]-PGE-M that the use of [$^{2}H_7$]-PGE-M allowed more reliable quantification of PGE-M and PGF-M than the use of [$^{2}H_3$]methoxime derivatives [150]. The precision of the method for PGE-M was 4.5% when [$^{2}H_7$]-PGE-M and 16% when [$^{2}H_3$]MO-PGE-M were used as internal standards. By using a similar procedure, this group reported on the determination of PGE-M, PGE_2 and five other prostanoids by GC–NICI-MS–MS in human urine [112]. PGE-M could be quantitated at an imprecision of less than 5% at urinary concentrations of about 1 ng/ml [112]. A detection limit of less than 50 pg for PGE-M and PGF-M was reported by this group [150]. The major drawback of the $^{2}H_7$-labelled analogs used is the poor isotopic purity because they contained substantial amounts of $^{2}H_2$- to $^{2}H_8$-species. For the determination of PGE-M in human urine by GC–NICI-MS–MS using a $^{18}O_2$-labelled analog, we developed a rapid method involving isocratic RP-HPLC separation of the PFB-MO derivatives of PGE-M (Fig. 5b), PGE_2 and other prostanoids [131]. The $^{18}O_2$-labelled PGE-M analog contained only 0.7% of unlabelled PGE-M. Imprecision and detection limit of our method were 3.5% and 10 pg/ml urine, respectively. The main advantages of this method is its practicality, rapidity, quantitative recovery of the PFB-MO derivative of PGE-M and other prostanoids from respective RP-HPLC fractions and the high degree of automation including fully programable autosamplers both for RP-HPLC and GC and sample collector for RP-HPLC fractions. In comparison with other prostanoids, PGE-M reveals the highest limit of detection. The lower sensitivity for PGE-M results from the formation of four GC incompletely resolved methoxime isomers, and additionally from the low volatility of the isomeric diPFB-diMO-TMS derivatives. This is reflected in the longest retention time on GC and in the broadest GC peaks of the most commonly analysed prostanoids. Because of the need of the use of at least one step for sample purification even in GC–NICI-MS–MS, the target of GC–MS methods for PGE-M should be the choice of the most dominant methoxime isomer [131].

A precise (7%), accurate (96%), sensitive (50 pg/mg creatinine) but labor-intensive and cumbersome GC–NICI-MS method for the quantification of PGD-M in human urine has been reported by Morrow et al. [135]. This group used $^{18}O_4$-labelled PGD-M as an internal standard in a method consisting of various derivatization and purification procedures including TLC, in particular taking advantage of the ability of the lower side chain of PGD-M to undergo cyclization at acidic pH to form a hemiketal (Scheme 5). This group have modified their original method so that the quantification of PGD-M in human urine by GC–NICI-MS became more rapid and simple as no purification by TLC was necessary (Fig. 5c) [109]. This group have reported that both the original and the modified assay revealed comparable imprecision (8.5%) and accuracy for increased levels of PGD-M in urine. However, as Fig. 5c shows, accurate analysis of PGD-M in human urine by the original as well as by the modified assay is rendered more difficult by unknown compounds, the derivatives of which have almost identical R_f values on TLC and identical $[M-PFB]^-$ anions. In the upper trace of the chromatograms at m/z 514, peak I suggests representation of PGD-M exclusively while most of peak II and all of peaks III and IV suggest representation of metabolites of non-COX-derived prostanoids (F_2-isoprostanes) [109].

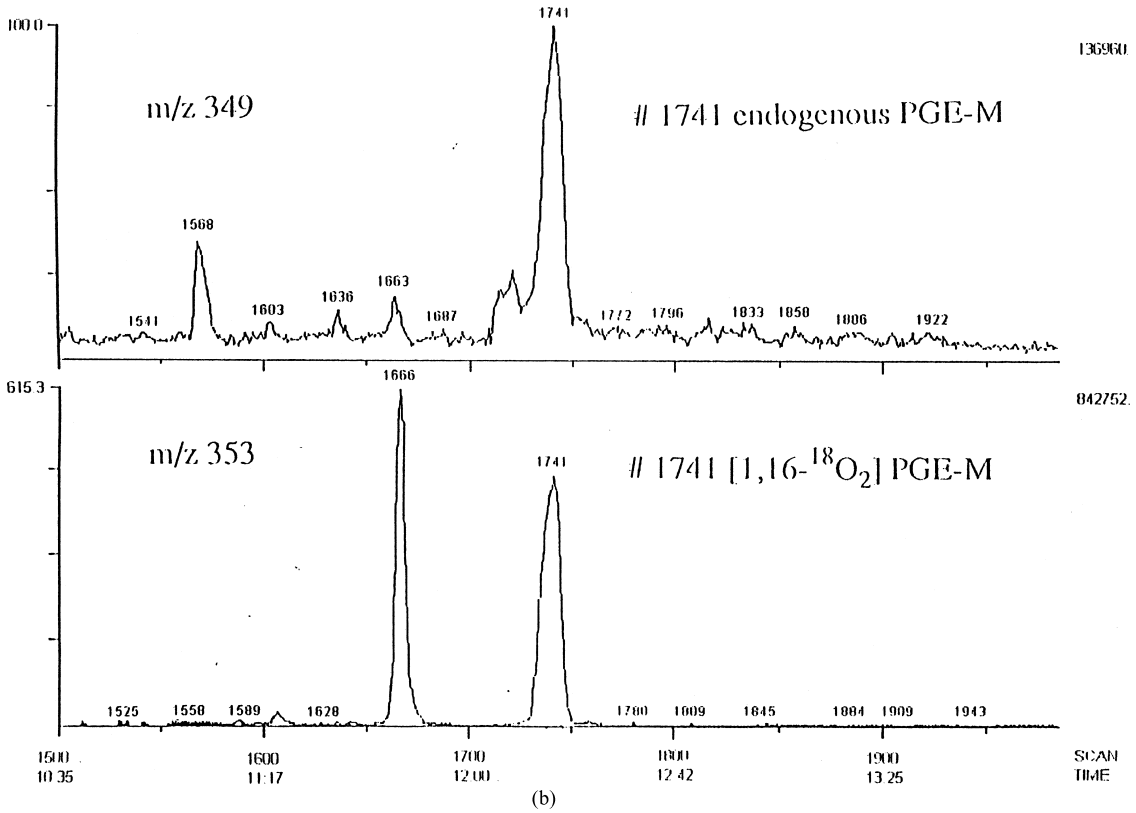
For the quantitative analysis of PGE_2, PGD_2 and PGF_2 in human urine and plasma and of circulating metabolites of them, various GC–MS methods have been developed [64,79,88,89,103] [112,114,131,160] [197,223,224,226,227].



(a)



(c)



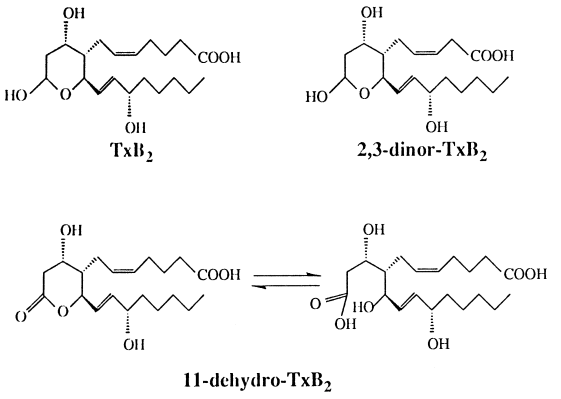
(b)

Fig. 5. Partial chromatograms from the analysis of PGF-M (a) and PGE-M (b) by GC–NICI-MS–MS and PGD-M (c) by GC–NICI-MS in human urine. PGE-M in (b) was analysed using [1,16-$^{18}O_2$]PGE-M as described in [131]. From [150] (a) and [109] (c) with permission. Upper traces show endogenous compounds and lower traces internal standards. For more details regarding PGD-M see Section 4.

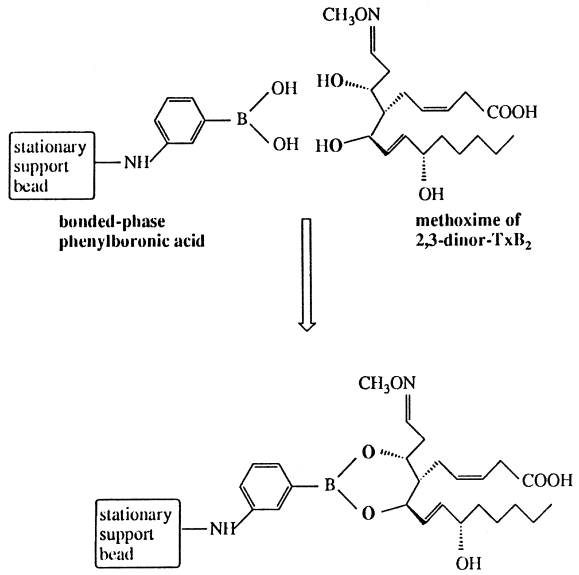
4.1.2. Thromboxane and index metabolites

In 1975, Hamberg et al. discovered the potent proaggregatory and vasoconstrictor thromboxane A_2 (TxA_2) in platelets [228]. At physiological pH, TxA_2 is extremely labile and nonenzymatically converted to TxB_2. Roberts et al. identified by GC–EI-MS, 2,3-dinor-TxB_2 as the major urinary metabolite of systemically administered TxB_2 in man [229]. Four years later, Roberts et al. identified by GC–EI-MS twenty urinary metabolites of TxB_2 among them 2,3-dinor-TxB_2 and 11-dehydro-TxB_2 were the most abundant [230] (Scheme 6). These studies have provided the background biochemical information necessary to develop methods for quantification of urinary 2,3-dinor-TxB_2 and 11-dehydro-TxB_2 as a measure of in vivo production of TxB_2 in man.

Maas et al. have reported the first GC–EI-MS method for the quantitative measurement of 2,3-dinor-TxB_2 in 50- to 100-ml aliquots of human urine [95]. This method required extensive sample purification to ensure precise (within 10%) analysis. For very low 2,3-dinor-TxB_2 levels as they occur in humans taking inhibitors of the COX enzyme, an additional purification by TLC was found to be necessary. One year later, Vesterqvist et al. reported an accurate (95%) GC–EI-MS method which also involved extensive purification steps requiring 10- to 40-ml aliquots of urine [163]. Great improvement in quantitative GC–MS analysis of TxB_2 and its metabolites in human urine was achieved by introducing a highly selective SPE of methoximated 2,3-dinor-TxB_2 and TxB_2 with PBA cartridges [155]. Lawson et al. have utilized the ability of the tetrahedral anionic form of boronates to condense with 1,2- or 1,3-diols to form five- or six-membered covalent complexes. They have shown that methoximation of 2,3-dinor-TxB_2 and TxB_2 in urine or aqueous solutions is necessary in order to convert these compounds to 1,3-diols that can selectively be extracted by PBA SPE cartridges (Scheme 7). Lawson et al. have reported that the open ring form of 11-dehydro-TxB_2 is also selectively extractable with PBA cartridges [155]. Despite selective extraction of 2,3-dinor-TxB_2 and TxB_2 from human urine, their quantification by GC–NICI-MS in this matrix required a TLC purification step (Fig. 6) [155]. Imprecision and accuracy of this method have been reported to be 6% and 95%, respectively. Lawson et al. have not reported on the suitability of the method for simultaneous quantification of 2,3-dinor-TxB_2 and TxB_2 in human urine [155]. The need of a TLC purification step for GC–NICI-MS determination of 2,3-dinor-TxB_2 and TxB_2 in human urine despite selective extraction by PBA cartridges has also been reported by Weber et al. [172]. By using a similar extraction procedure for urinary 2,3-dinor-TxB_2 and TxB_2 on PBA cartridges to that reported by Lawson



Scheme 6. Chemical structures of TxB_2, 2,3-dinor-TxB_2 and 11-dehydro-TxB_2.



Scheme 7. The methoxime derivative of 2,3-dinor-TxB_2 contains a 1,3-diol that condensates with bonded-phase phenylboronic acid (PBA) to form a stable complex.

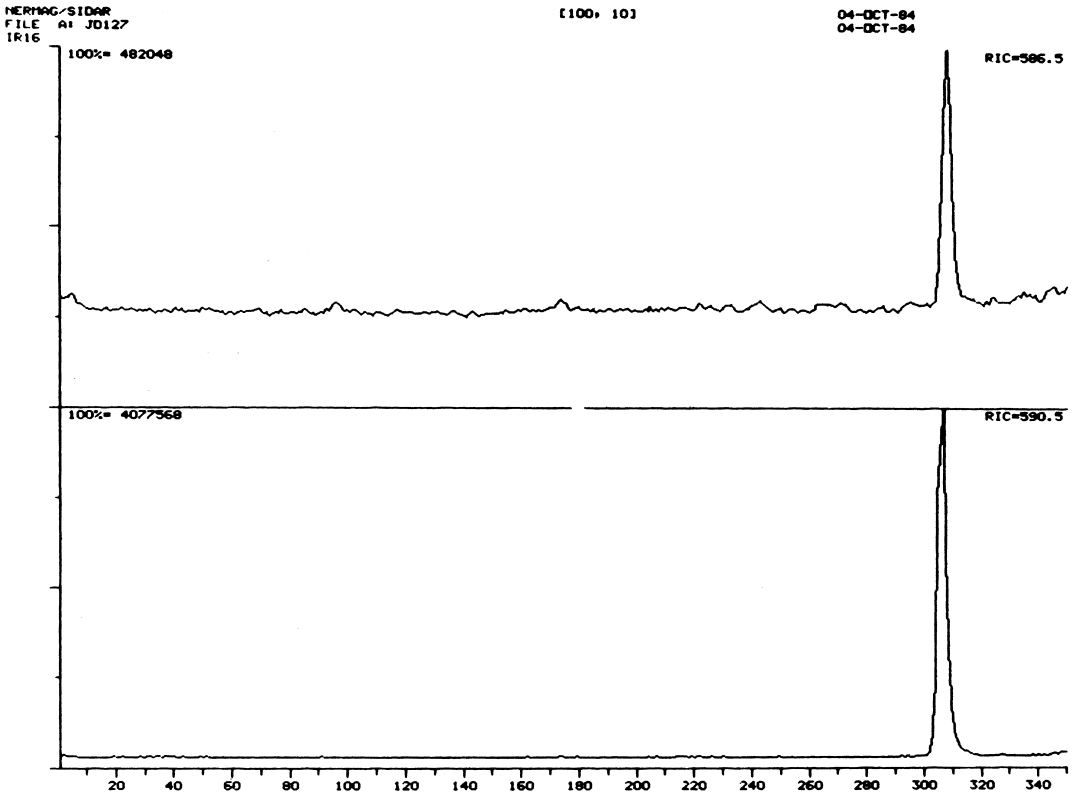


Fig. 6. Analysis of 2,3-dinor-TxB_2 in human urine by GC–NICI-MS methods based on SPE by PBA cartridges and TLC as described by Lawson et al. [155]. Upper trace shows endogenous, the lower trace the deuterated internal standard. From [155] with permission.

et al. [155], we found that quantification of 2,3-dinor-TxB_2 in 5-ml aliquots of human urine can be accomplished by GC–NICI-MS–MS without the need of further purification steps like TLC or HPLC (Fig. 7a) [185]. Moreover, we found that the combination of this method with that of Daniel et al. [111] described for 2,3-dinor-6-oxo-$PGF_{1\alpha}$ allows simultaneous quantification of 2,3-dinor-TxB_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ (Fig. 7b; see also Fig. 4 and Scheme 8) [185]. Simultaneous measurement of 2,3-dinor-TxB_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ as their PFB-MO-TMS derivatives at maximum sensitivity for each compound is possible because of the complete GC separation and identical parent and daughter ions (Table 2). Imprecision of this method for the simultaneous measurement of 2,3-dinor-TxB_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in human urine at concentrations of 40 to 300 pg/ml was determined as 5.9% and 4.6%, respectively. Accuracy of the method within this range was 95% for 2,3-dinor-TxB_2 and 98% for 2,3-dinor-6-oxo-$PGF_{1\alpha}$. A simple and rapid method for quantitation of 2,3-dinor-TxB_2 and TxB_2 in human urine by GC–NICI-MS based on immunoaffinity extraction has been reported by Chiabrando et al. [159]. In this method immunoaffinity extracts were derivatized and analysed by GC–NICI-MS without any other purification. Chiabrando et al. [157] and Hubbard et al. [158] have reported rapid quantitative analysis of 2,3-dinor-TxB_2 and TxB_2 in human urine by high-resolution GC–NICI-MS utilizing a single antibody-mediated extraction procedure. Accuracy and imprecision of the assay of Chiabrando et al. [157] have been reported to be 97% and 2–10% for TxB_2, and 95% and 2–7% for 2,3-dinor-TxB_2, respectively.

Special interest has been attributed to 11-dehydro-TxB_2 because this metabolite has been shown to be an appropriate quantitative index metabolite of TxA_2

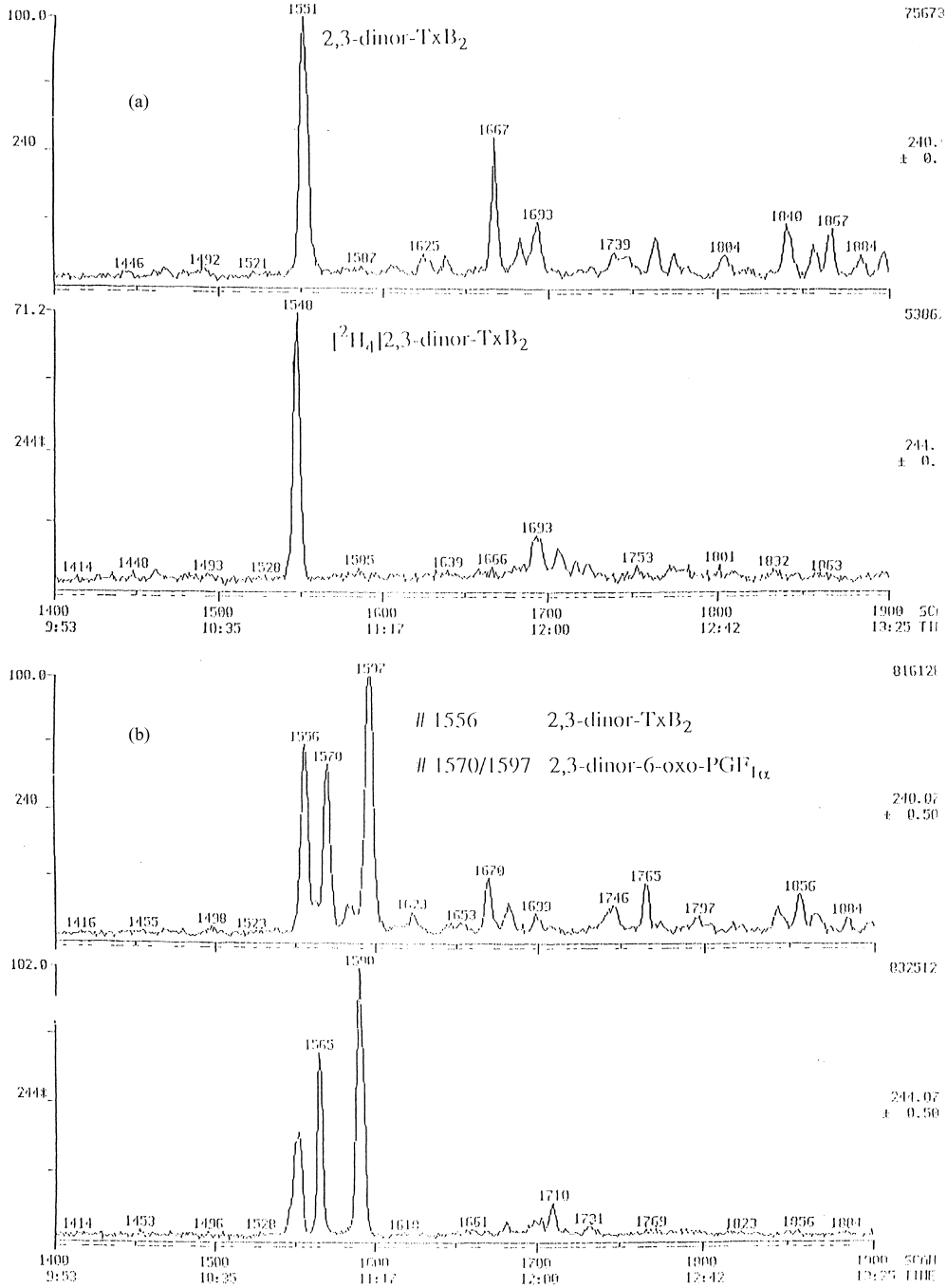
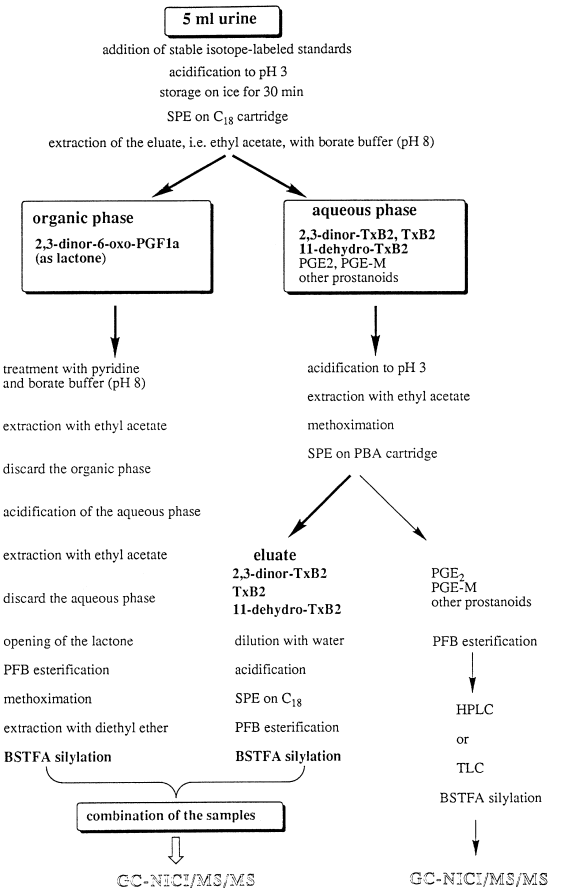


Fig. 7. (a) Analysis of 2,3-dinor-TxB_2 in human urine by GC–NICI-MS–MS using a similar procedure to that described by Lawson et al. [155] but without any other purification step [185]. Analyses of the separate fractions from the same urine sample prior to combination are shown in this figure. (b) Simultaneous analysis of 2,3-dinor-TxB_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in human urine by GC–NICI-MS–MS [185]. Urine samples were treated as described by Lawson et al. [155] for 2,3-dinor-TxB_2 and by Daniel et al. [111] for 2,3-dinor-6-oxo-$PGF_{1\alpha}$. Separate fractions were combined prior to GC–NICI-MS–MS analysis. Upper and lower traces show the endogenous compounds and the internal standards, respectively. For separate analysis of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in the same urine sample see Fig. 4.



Scheme 8. Summary of the extraction and purification steps for the simultaneous quantitative determination of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 2,3-dinor-TxB_2 in human urine by GC–NICI-MS–MS [185]. This procedure can also be used for the quantitation of other prostanoids when the respective fraction is combined with further purification steps such as HPLC or TLC.

both in plasma [180,190] and in urine [142]. Schweer et al. have developed a precise GC–NICI-MS–MS method for the determination of 11-dehydro-TxB_2 in human urine and plasma using a TLC purification step of the PFB ester derivatives [142]. Imprecision of the method for urinary 11-dehydro-TxB_2 was reported to be 5%. 11-Dehydro-TxB_2 levels in human plasma obtained by their less time-consuming GC–NICI-MS–MS method were comparable to results by Catella et al. who have used a TLC step more in their GC–NICI-MS method [180]. The urinary excretion of 11-dehydro-TxB_2 has been shown to be two times that of 2,3-dinor-TxB_2 [142]. This result differs from infusion studies [229], which have identified 2,3-dinor-TxB_2 as the major urinary metabolite of TxB_2.

For specific quantification of 11-dehydro-TxB_2 in human urine, several methods have been developed [110,134,171,182,183]. Lorenz et al. have reported that quantification of 11-dehydro-TxB_2 in human urine can simply be performed by a single extraction of 11-dehydro-TxB_2 on PBA cartridges and followed analysis by GC–NICI-MS–MS of the PFB-TMS derivatives without the need of any further purification step [171]. Imprecision and accuracy of this method were reported as 11% and 95%. Despite quantification by GC–NICI-MS–MS, Ferretti et al. have used a more laborious method which involved SPE, solvent extraction and TLC purification of Me-PFB-TMS derivatives [183]. The authors of this work have reported an imprecision of 1.3% and an accuracy of 85% for their method. Weber et al. have used PBA extraction of the open ring form of urinary 11-dehydro-TxB_2 for quantitation of the PFB-TMS derivatives by GC–NICI-MS but their method required only a single TLC purification step (Fig. 8) [182]. Imprecision, accuracy and detection limit of this assay have been reported as 2.5%, 101%, and 10 pg/ml, respectively. Morrow and Minton [110] have modified and improved the original GC–MS method of Lawson et al. [190]. Morrow and Minton have reported that their assay is characterized by an imprecision of 7%, an accuracy of 90% and a

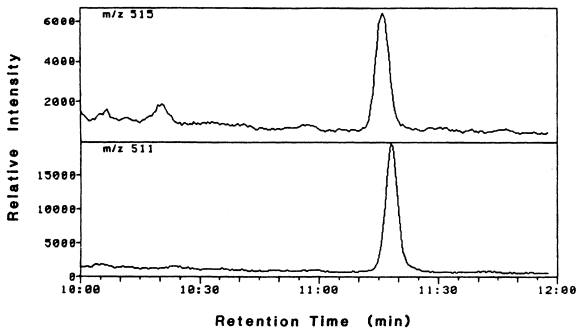


Fig. 8. Analysis of 11-dehydro-TxB_2 in human urine by GC–NICI-MS as its PFB–TMS derivative. Extraction of the open ring form of 11-dehydro-TxB_2 from urine was performed employing PBA cartridges. A TLC step was used for purification. Upper and lower traces show the internal standard and the endogenous compound, respectively. With permission of [182].

detection limit of 20 pg/mg creatinine. Nevertheless, their method is still time-consuming because it contains two TLC purification steps.

For the quantitation of 2,3-dinor-TxB_2, 11-dehydro-TxB_2 and TxB_2 in human urine and plasma, various GC–MS methods have been reported which require substantial purification prior to MS analysis [112,131,159,168,169,174,181].

4.1.3. Isoprostanes

In 1970, Hamberg and Israelsson identified 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ as PGE_2 metabolites in the soluble fraction of guinea pig liver homogenates [231]. Twenty years later, Morrow et al. observed that arachidonyl-containing lipids in plasma readily underwent autooxidation in vitro, resulting in the formation of a series of PGF_2-like, COX-independent compounds [96]. In 1990, Morrow et al. discovered by GC–MS, for the first time, production of PGF_2-like, COX-independent, isoprostane-termed compounds in vivo in humans and in CCl_4-treated rats [115]. Four years later, Morrow and co-workers reported evidence by GC–MS and HPLC that one of these F_2-isoprostanes, namely 8-iso-$PGF_{2\alpha}$, is formed in vivo in the rat [117]. The same group have reported evidence by GC–MS for the occurrence in human urine and plasma of a non-COX-derived F-ring metabolite which has been suggested to be isomeric to PGD-M [116]. However, this work did not present unequivocal evidence for iso-PGD-M. Morrow et al. have also reported on free radical-induced generation of D-ring and E-ring isoprostanes in vivo [232]. One of the E_2-isoprostanes was expected to be 8-iso-PGE_2. Roberts et al. have recently identified, by GC–MS, 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ as the major urinary metabolite of 8-iso-$PGF_{2\alpha}$ in humans from intravenously infused tritiated 8-iso-$PGF_{2\alpha}$ [233]. Interestingly, this metabolite is distinct from the PGF-M, the major urinary metabolite of $PGF_{2\alpha}$ in humans [221]. Morrow et al. have also provided evidence for nonenzymatic free radical-catalyzed formation of several iso-thromboxanes in vivo in the rat [234]. Whether one of these iso-thromboxanes is 8-iso-TxB_2 is unclear. Today, no data exist on the occurrence of iso-thromboxanes, iso-prostacyclin and metabolites of them in human plasma or urine. Also, the metabolic fate of these and other isoprostanes in humans is unsatisfactorily or completely unknown.

In addition to its generation by free radical-catalyzed mechanisms, 8-iso-$PGF_{2\alpha}$ has been shown to be formed by the catalytical action of the COX but at a amount of about two orders of magnitude smaller [235–237]. It is, therefore, generally accepted that isoprostanes are novel markers of free radical-catalyzed lipid peroxidation and potential mediators of oxidant injury [97,238]. This opinion mainly originated from observations of GC–MS measurements of F_2-isoprostanes as a group or as a not yet unequivocally structurally identified urinary metabolite of F_2-isoprostanes rather than from assessment of a particular isoprostane such as 8-iso-$PGF_{2\alpha}$ [91,239–241]. However, the recognition of 8-iso-$PGF_{2\alpha}$ as an abundant isoprostane in the urine of the rat [117] has prompted many groups to develop methods for the assessment of this isoprostane in human plasma and urine.

Wang et al. have characterized and quantitated 8-iso-$PGF_{2\alpha}$ in human urine by EIA and RIA [71]. These assays were validated by GC–NICI-MS using [$^{18}O_2$]-8-iso-$PGF_{2\alpha}$ as an internal standard. The GC–MS method involved a SPE extraction from urine on C_{18} cartridges and two TLC purification steps. Nourooz-Zadeh et al. have developed a method for the quantitative determination of 8-iso-PGF_{2a} in human plasma by GC–NICI-MS using [$^2H_4$]-$PGF_{2\alpha}$ as internal standard [119]. Unesterified free 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ from alkaline hydrolysis of esterified 8-iso-$PGF_{2\alpha}$ were extracted from plasma by SPE on C_{18} cartridges followed on NH_2 cartridges. By using an SPB-1701 capillary column, base-line separation of the PFB-TMS derivatives of 8-iso-$PGF_{2\alpha}$ from the corresponding derivatives of $PGF_{2\alpha}$, 9a,11β-PGF_2 and 9b,11α-PGF_2 was achieved. Free 8-iso-$PGF_{2\alpha}$ was not detectable in plasma by this method while the sum of free and esterified 8-iso-$PGF_{2\alpha}$ was reported to be 58 to 166 pg/ml plasma [119]. Evidence for the existence of esterified F_2-isoprostanes has been demonstrated by FAB-MS–MS [242]. Bachi et al. have developed a rapid method for the measurement of 8-iso-$PGF_{2\alpha}$ in human urine by GC–NICI-MS using commercially available [$^2H_4$]-8-iso-$PGF_{2\alpha}$ as internal standard [120]. 8-iso-$PGF_{2\alpha}$ was extracted and purified in one step from human urine on immunoaffinity columns

prepared with an anti-8-iso-$PGF_{2\alpha}$ antiserum. Imprecision and accuracy of this assay were reported as 4.8% and 100.5%, respectively. Bachi et al. have measured by their method, significantly higher excretion rates of 8-iso-$PGF_{2\alpha}$ in smokers compared to nonsmokers [120]. Analogous to COX-derived prostanoids [157,159], Chiabrando and his group have shown that immunoaffinity extraction is a very efficient method to extract isoprostanes from human urine allowing quantification by simple GC–NICI-MS [120]. Quantitation of 8-iso-$PGF_{2\alpha}$ in human urine has also been achieved by GC–NICI-MS–MS using [$^{18}O_2$]-8-iso-$PGF_{2\alpha}$ [121] and commercially available [$^{2}H_4$]-8-iso-$PGF_{2\alpha}$ [123] as internal standards. For quantitation by GC–NICI-MS–MS, we found that a single TLC purification step for the PFB derivatives of 8-iso-$PGF_{2\alpha}$ is satisfactory to accurately measure urinary 8-iso-$PGF_{2\alpha}$ (Fig. 9) [123]. We observed the best GC separation of 8-iso-$PGF_{2\alpha}$ from $PGF_{2\alpha}$ and other PGF_2-isomers by using a SPB-1701 capillary column. By using this method we found highly elevated 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ levels in urine of children with Zellweger syndrome as compared to healthy children [123]. Our method showed an imprecision of 7% and an accuracy of 95% at urinary levels of 130 pg/ml. We were able to accurately detect as much as 5 pg/ml of [$^{2}H_4$]-8-iso-$PGF_{2\alpha}$ added to a 5-ml aliquot of a human urine sample. Ferretti and Flanagan have also developed a GC–NICI-MS method for urinary 8-iso-$PGF_{2\alpha}$ [122]. They reported that sample cleanup in their method required the combined use of HPLC for the free acids and TLC for the PFB esters. Without the use of HPLC, no acceptable accuracy was obtained by this group. Furthermore, Ferretti and Flanagan reported that the use of GC–NICI-MS–MS could not further improve the specificity of the method [122]. Imprecision of this method for urinary levels of 1.26

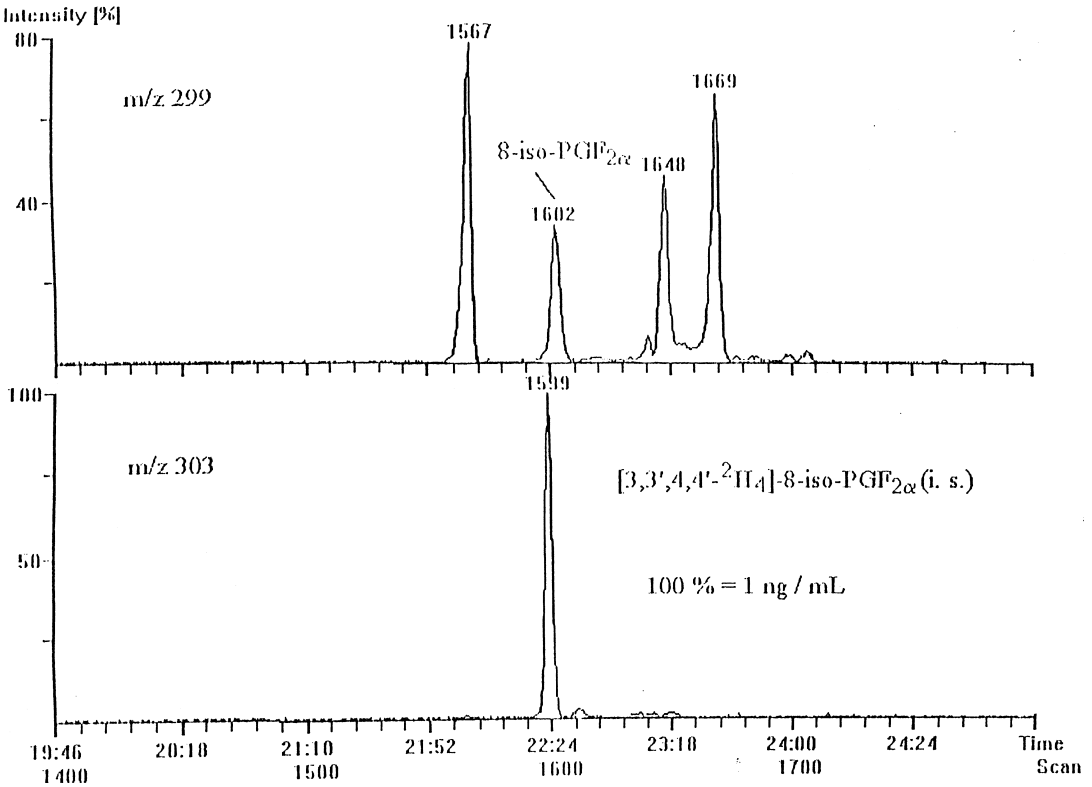


Fig. 9. Partial chromatogram from the quantitative GC–NICI-MS–MS determination of 8-iso-$PGF_{2\alpha}$ in human urine. From Ref. [123] with permission.

ng/ml and 0.87 ng/ml has been reported to be 1.6% and 2.3%, respectively. Accuracy and limit of detection of the method were given as 91% and 25 pg/ml, respectively.

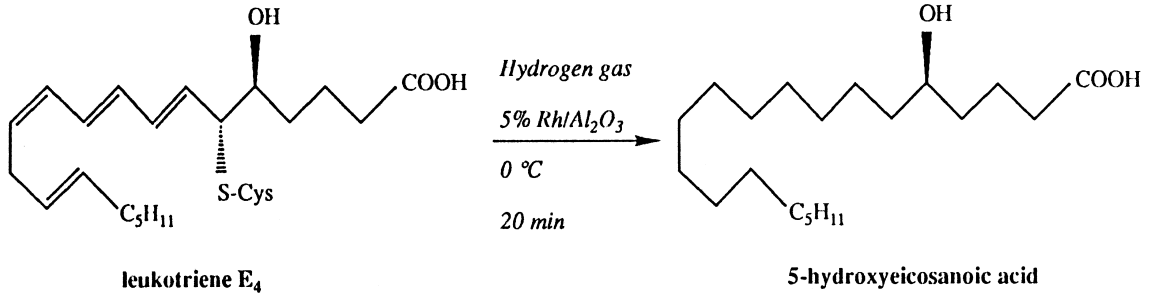
It should be noted that because of readily formation of isoprostanes by autooxidation ex vivo in lipid-containing biological samples especially in plasma, appropriate precautions must be taken to prevent artifactual generation of isoprostanes [238]. Butylated hydroxytoluene and triphenylphosphine have been shown to be suitable antioxidants for such purposes [91]. Also, the use of a combination of 5-hydroxy-tempo with EDTA did not show any variation of the levels of 8-iso-$PGF_{2\alpha}$ in human urine over a 6-months period when samples have been stored at −20°C [71].

4.2. Leukotrienes

4.2.1. Cysteinyl leukotrienes

The 'slow reacting substance of anaphylaxis' (SRS-A) has been discovered during anaphylactic reactions already in 1930s. Structural elucidation of LTC_4 was reported in 1979 [7,243,244]. Treatment of SRS-A with Raney nickel and hydrogen gas to remove the sulphur-containing moiety resulted in an ether-extractable product that was identified by GC–MS as 5-HEA [243]. This indicated that the SRS-A was an eicosanoid containing a hydroxyl group at C-5. The simultaneous catalytical saturation of the double bonds and the desulphurisation of the cysteinyl moiety of cysteinyl leukotrienes by noble catalysts and hydrogen gas in a two-step procedure to yield quantitatively 5-HEA was reported first by Murphy's group (Scheme 9) [52,53,106]. Best results were obtained by using 5% rhodium on alumina as the catalyst. Hydrogenation of methanolic solutions of LTC_4, D_4, and E_4 for 20 min at 0°C was shown to yield in one step about 80% of 5-HEA [106]. This method was demonstrated to be suitable for the quantitative determination of the cysteinyl leukotrienes produced by rat lung and mouse peritoneal macrophages by GC–NICI-MS using [$^{18}O_2$]-LTE_4 or [$^{18}O_2$]-5-HETE as internal standards [52,53,106]. The internal standards were added to the respective HPLC fractions of the cysteinyl leukotrienes prior to catalytical hydrogenation. Current GC–MS methods for quantitation of cysteinyl leukotrienes in human urine are based on the catalytical saturation/desulphurisation described by Balazy and Murphy [106]. The first application of this method for the identification of LTE_4 in human urine by GC–MS was demonstrated by Fauler et al. [66]. Other mass spectrometric techniques of intact and derivatized cysteinyl leukotrienes still containing the cysteinyl moiety has also been applied but they are not suitable for quantitation in biological fluids, mainly due to lack in sensitivity [reviewed in [7]].

In 1985, Örning et al. investigated in vivo metabolism of injected tritiated LTC_4 in man [245]. In this study, LTE_4 was found to be the major metabolite of LTC_4 in urine. Verhagen et al. have found that LTE_4 was the major urinary metabolite of LTD_4 inhaled by healthy humans [246]. Also, Maltby et al. found that infused radiolabelled LTC_4 in man was excreted mainly as LTE_4 into the urine [247]. Huber et al. have found similar results in monkey and in man [248]. These studies provided evidence for LTE_4 as the major urinary metabolite of exogenous cysteinyl leukotrienes in man suggesting, analogous to urinary



Scheme 9. Catalytical reduction and desulphurization of LTE_4 to yield 5-hydroxyeicosanoic acid (5-HEA). Its derivatization to the PFB ester – TMS ether derivative allows GC–MS quantitation of LTE_4.

metabolites of prostanoids, that LTE_4 may be a useful index metabolite for whole body production of cysteinyl leukotrienes. On the other hand, Sala et al. have found that infused radiolabelled LTE_4 into healthy humans was excreted almost unmetabolized in the first urine collection, while later two more polar compounds were found predominately in the urine [178]. These compounds were identified by GC–MS and GC–tandem MS as 14-carboxy-hexanor-LTE_3 and 16-carboxy-Δ^{13}-tetranor-LTE_4. 16-Carboxy-tetranor-LTE_3, 18-carboxy-dinor-LTE_4 and 20-carboxy-LTE_4 were found to be minor metabolites of LTE_4 in the urine [178]. Sala et al. have suggested that the major urinary metabolites of LTE_4, namely 14-carboxy-hexanor-LTE_3 and 16-carboxy-Δ^{13}-tetranor-LTE_4, might better reflect total body production of cysteinyl leukotrienes in vivo in man, because measurement of these metabolites would eliminate potential problems associated with kidney LTE_4 production and elimination [178]. Nevertheless, today urinary LTE_4 is generally accepted as the most useful index parameter to assess in vivo formation of cysteinyl leukotrienes in man. The almost exclusive use of urinary LTE_4 as a measure of cysteinyl leukotriene production in humans most likely originates from the more simple measurement of urinary LTE_4 by various analytical techniques. Based on the measurement of urinary excretion rates of LTE_4 in vivo in humans, cysteinyl leukotrienes were found to be implicated in many diseases such as asthma [48,67,72,249], cystic fibrosis [65], multiple trauma with or without adult respiratory distress syndrome [66], active systemic lupus erythematosus [68], atopic dermatitis [105], psoriasis [205], juvenile rheumatoid arthritis [206], and liver cirrhosis and hepatorenal syndrome [57].

Usually, urinary LTE_4 is measured by RIA or EIA following separation by HPLC [33,48,59,61,62] [65–69,72,73,77] [105,205,206]. Mainly because of lack in sensitivity and selectivity, urinary LTE_4 cannot be quantitated by a combination of HPLC with UV or fluorescence [36,37]. The application of these HPLC methods is exclusively limited to in vitro measurements [29,35,58]. Unlike in prostanoids research, GC–MS could not gain prevalence in the quantitation of cysteinyl leukotrienes in human urine. Many of the HPLC–RIA and HPLC–EIA offered very similar values for the excretion rates of LTE_4 in healthy humans to that measured by GC–tandem MS [129,132,151]. However, especially in the face of diseased humans, the many biochemical abnormalities encountered make it mandatory to assess LTE_4 additionally by GC–MS [66,105,205,206]. The HPLC–RIA method used in our group was validated by GC–NICI-MS–MS. We found a very close correlation for urinary levels of LTE_4 by the two methods [105]. Also, very similar urinary levels were found by both methods in patients with psoriasis and multiple trauma [66,204,205].

With the exception of the absolute necessity to use stable isotope-labelled cysteinyl leukotrienes as internal standards and catalytical hydrogenation to obtain volatile derivatives, GC–MS and HPLC–RIA or HPLC–EIA methods for specific measurement of urinary cysteinyl leukotrienes involve almost the same procedures for extraction, purification and separation. A critical point in the GC–MS quantitation of cysteinyl leukotrienes is the election of a suitable internal standard for a particular cysteinyl leukotriene. Before deuterium-labelled cysteinyl leukotrienes became commercially available, we synthesized enzymatically [1,1-$^{18}O_2$]LTE_4 in high isotopic purity and demonstrated its excellent suitability as an internal standard for quantitative determination of urinary LTE_4 by GC–NICI-MS–MS [105,129]. Later, [20,20,20-$^2H_3$]LTE_4 became commercially available [108]. In a study, we tested the suitability of [20,20,20-$^2H_3$]LTE_4 and of a self-synthesized [14,15,17,17,18,18-$^2H_6$]LTE_4 [146] as internal standard for LTE_4 in comparison to [1,1-$^{18}O_2$]LTE_4 [151]. Among them [1,1-$^{18}O_2$]LTE_4 was found to be the best suited internal standard. ^2H-Exchange during catalytical hydrogenation occurred both in [20,20,20-$^2H_3$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4. However, the lower extent of ^2H-exchange in [20,20,20-$^2H_3$]LTE_4 allowed more precise quantitation than the use of [14,15,17,17,18,18-$^2H_6$]LTE_4. The method permitted quantification of LTE_4 in human urine at imprecision values of 6%, 5% and 15% when [20,20,20-$^2H_3$]LTE_4, [1,1-$^{18}O_2$]LTE_4 and [14,15,17,17,18,18-$^2H_6$]LTE_4 were used as internal standard, respectively [151]. In [14,15,17,17,18,18-$^2H_6$]LTE_4 four ^2H-atoms stay at sp^2-C atoms and are apparently more activated during the catalytical hydrogenation process than those staying at sp^3-C atoms. These results suggested

that ^{2}H-labelled cysteinyl leukotrienes at saturated carbons atoms may be thoroughly suitable for use as internal standards in GC–MS methods.

To date, no stable isotope-labelled ω-carboxy-LTE_4 are commercially available. Mayatepek et al. prepared enzymatically a $^{18}O_2$-labelled ω-carboxy-LTE_4 starting from unlabelled ω-carboxy-LTE_4 and showed its utility for quantitation of urinary ω-carboxy-LTE_4 in urine of children by GC–NICI–MS [69]. Interestingly, ^{18}O-incorporation into ω-carboxy-LTE_4 was catalyzed by butyryl cholinesterase exclusively in the carboxylic group at the ω-end. Our group has described chemical synthesis of [1,20-$^{18}O_2$]carboxy-LTE_4, [1,18-$^{18}O_2$]carboxy-dinor-LTE_4 and [1,16-$^{18}O_2$]carboxy-14,15-dihydro-tetranor-LTE_4 starting from the unlabelled dimethylesters of 20-carboxy-LTA_4, 18-carboxy-dinor-LTA_4 and 16-carboxy-14,15-dihydro-tetranor-LTA_4, respectively [132]. In this work, we demonstrated that these $^{18}O_2$-labelled ω-carboxylated LTE_4 metabolites are suitable internal standards for quantification of the corresponding endogenous compounds in human urine (Fig. 10). The imprecision of the method was determined as 5% for LTE_4 and 6% for 20-carboxy-LTE_4 at basal urinary levels of 20 and 16 nmol/mol creatinine, respectively. In healthy children and normal healthy adults, we measured by GC–NICI–MS–MS, comparable excretion rates for LTE_4 and 20-carboxy-LTE_4. Elevated LTE_4 (by HPLC–RIA) and 20-carboxy-LTE_4 (by GC–NICI-MS) excretion rates were measured in children with Zellweger syndrome [69]. On the other hand, 16-carboxy-14,15-dihydro-tetranor-LTE_4 was not detectable (by HPLC–RIA) in the urine of the diseased children but only in healthy infants controls [69].

It has been reported that untreated urine samples can be stored for several months at −20°C without significant loss of LTE_4 [72]. As a rule, however, pH adjustment to 9 and more frequently addition of antioxidants such as 4-hydroxy-TEMPO (1 mmol/l) and EDTA (0.5 mmol/l) to urine samples for the purpose of storage avoid oxidation of LTE_4 and of other cysteinyl leukotrienes during sample collection and storage [48,59,62,66,69,77,248].

Convenient extraction of cysteinyl leukotrienes from slightly acidified (pH 3 to 5.4) human urine samples is usually carried out on C_{18} cartridges [21,48,66,69,72,132]. Kikawa et al. have reported that the combined use of C_{18} and NH_2 cartridges for the SPE of urinary LTE_4 improved the measurement of LTE_4 by HPLC–RIA [61]. They obtained significantly lower levels for urinary LTE_4 when the combination of C_{18} with NH_2 cartridges was used. This result was interpreted as an improvement of the specificity of the method due to elimination of LTE_4-like immunoreactive material by the NH_2 cartridges. We also found that combined SPE of urinary LTE_4 with C_{18} and NH_2 cartridges makes the matrix more clear as was seen by RP-HPLC of a urinary extract and detection at 280 nm (unpublished observations). Using the extraction procedures described by Kikawa et al. [61], we were able to separate LTE_4 almost completely from LTD_4 and LTC_4. Nevertheless, the combined SPE with C_{18} and NH_2 cartridges did not sufficiently improve selectivity so that the use of subsequent purification by RP-HPLC was necessary prior to analysis by GC–NICI-MS or GC–NICI-MS–MS.

RP–HPLC is an indispensable chromatographic step for selective analysis of LTE_4 and other cysteinyl leukotrienes in human urine even by the use of GC–NICI-MS–MS. This absolute necessity stems from impurities in the sample and from structurally closely related compounds, the catalytical hydrogenation of which will alternately yield 5-HEA or other isomers of 5-HEAs and carboxylated 5-HEA and its isomers. Co-elution of the PFB-TMS derivatives of these compounds with the PFB-TMS derivative of LTE_4 or with those from its carboxylated metabolites on GC capillary columns would yield inaccurate values for LTE_4 and its carboxylated metabolites when measured by GC–NICI-MS. The use of GC–NICI-MS–MS eliminates interferences originating from isomers but this technique is not able to leave out of account interfering substances carrying a hydroxy group at C-5. As a rule, separation of urinary LTE_4 and its carboxylated metabolites by RP-HPLC is accomplished by gradient elution using secondary or ternary mixtures [66,69,132]. Mobile phases consisting of acetonitrile/methanol/water/acetic acid of pH 5.4 were found to be the most suitable for complete separation of a series of cysteinyl leukotrienes, LTB_4 and their metabolites of ω- and β-oxidation [132]. Despite use of high-resolving RP-HPLC and GC–NICI-MS–MS, 18-carboxy-dinor-LTE_4 and 16-carboxy-14,15-

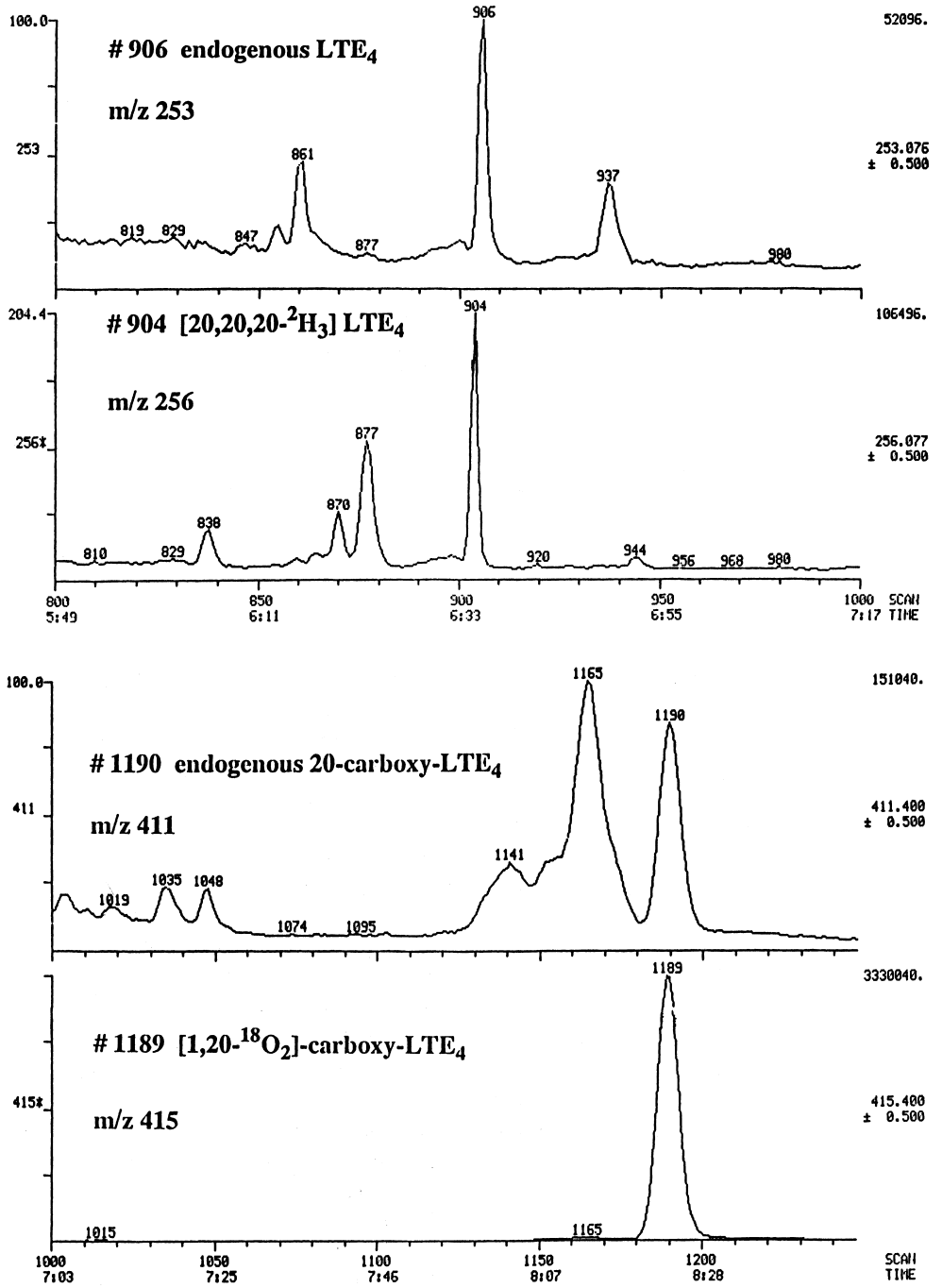


Fig. 10. Partial GC–NICI-MS–MS chromatograms from the analysis of LTE_4 (top) und 20-carboxy-LTE_4 (bottom) in human urine using [20,20,20-$^2H_3$]LTE_4 and [1,20-$^{18}O_2$]carboxy-LTE_4 as internal standards, respectively. From Ref. [132] with permission.

dihydro-tetranor-LTE_4 could not accurately be measured in human urine [132]. Reliable quantitation of these LTE_4 metabolites in human urine by GC–MS requires more efficient HPLC separation from numerous closely related compounds derived from C_{20}-, C_{18}- and C_{16}-hydroxylated fatty acids and other unknown interfering substances [132]. Efficient chromatographic separation could enable simultaneous quantitation of LTE_4 and its ω-carboxylated metabolites in human urine by GC–NICI-MS–MS [132].

Catalytical hydrogenation is easily performed by bubbling hydrogen gas in a conus glass vial containing a solution of cysteinyl leukotrienes in 1 ml of methanol and 5 mg of 5 wt.% of Rh/Al_2O_3 for 20 min at 0°C [129]. After this process, the sample is centrifuged and the catalyst is washed with 1 ml of methanol. The combined methanol supernatants are evaporated under nitrogen, the residue is dissolved in 1 ml of water and the reaction product 5-HEA is extracted twice with 1 ml of ethyl acetate. At this step, acidification must be avoided because of easy formation of the δ-lactone of 5-HEA [191]. The solvent is dried over anhydrous sodium sulphate and then completely removed under nitrogen. Reaction products are converted to their PFB-TMS derivatives by standard derivatization procedures.

4.2.2. Leukotriene B_4

In 1979, at the same time as LTC_4 was discovered, Borgeat and Samuelsson discovered LTB_4 as a dihydroxy metabolite of arachidonic acid by rabbit peritoneal polymorphonuclear leucocytes, the structure of which was elucidated to be 5,12-dihydroxy-eicosatetraenoic acid by mass spectrometry of the native and of the hydrogenated metabolite [7,250,251]. Twenty-six years later, Harrison and Murphy discovered the biologically active B_4-iso-leukotrienes as 5-LO independent, free radical-catalyzed oxidative products of glycerophospholipids [252]. Rabbit peritoneal polymorphonuclear leucocytes have been shown to additionally form two stereoisomers of LTB_4, i.e. 6-trans-LTB_4 and 6-trans-12-epi-LTB_4, and two positional isomers, i.e. 5,6-dihydroxy-7,9,11,14-eicosatetraenoic acids, epimeric at C-6 [250]. In vitro, LTB_4 is metabolized via distinct metabolic pathways to a variety of biologically less active or completely inactive products [24,25,28,49,204,253–256] [257–259]. The most significant metabolic fate of LTB_4 involves ω-oxidation to 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 followed by β-oxidation from the ω-end. Keppler's group have shown that both 20-carboxy-LTB_4 and the 20-carboxy-*N*-acetyl-LTE_4 are β-oxidized exclusively from the ω-end in peroxisomes in vitro [260]. Since in vivo in children with peroxisome deficiency syndrome, no β-oxidized metabolites of LTB_4, cysteinyl leukotrienes, prostaglandins and thromboxane are excreted into the urine, β-oxidation of these eicosanoids in vivo in man seems to exclusively take place in the peroxisomes [69,131].

In contrast to prostanoids and cysteinyl leukotrienes, LTB_4 undergoes in vivo rapid and extensive metabolism via the β-oxidation system. Serafin et al. have studied in vivo metabolism of tritiated LTB_4 in the monkey [192]. They found that more than 70% of infused radiolabelled LTB_4 was converted to tritiated water. The major nonvolatile urinary metabolite of LTB_4, representing 0.8% of the infused material, was identified as the δ-lactone of 20-hydroxy-LTB_4 while unchanged LTB_4 was not found in the urine. In the same study similar results with infused LTB_4 and 8,15-dihydroxy-HETE in rabbits were observed.

Today only limited information is available concerning the ultimate metabolic fate of LTB_4 in vivo in humans. In urine samples of patients with Zellweger syndrome, we identified by GC–NICI-MS–MS endogenous LTB_4 and 20-carboxy-LTB_4 [69]. By contrast, in the urine of healthy infant controls neither LTB_4 nor 20-carboxy-LTB_4 could be discovered. Thus, from the data available today, neither LTB_4 and 20-carboxy-LTB_4 nor other metabolites of them are suitable index metabolites in urine to assess synthesis of LTB_4 in vivo in humans. The lack of suitable LTB_4 metabolites in human urine alerted interest in other LTB_4-containing biological fluids such as plasma or serum and synovial fluid.

LTB_4 has been frequently quantitated in human serum or plasma and synovial fluid by RIA or EIA and by GC–MS methods. However, GC–MS has found a wider applicability in the analysis of LTB_4 than of LTE_4. Immunoassays and GC–MS methods for LTB_4 are based on procedures for SPE and HPLC which resemble or are identical with those for LTE_4 and its metabolites [69,102,113,179,207]. HPLC with UV detection has been widely employed in studies in isolated cell systems, but this technique

did not find any application for endogenous levels of LTB_4 in human plasma or synovial fluid. Today very few data are available for measurement of endogenous levels of LTB_4 in plasma. Blair and colleagues have developed a GC–NICI-MS method for the quantitative determination of LTB_4 in human serum using $[^2H_2]LTB_4$ as internal standard [179]. This method involved SPE of LTB_4 from acidified serum and two sequential TLC steps for the free acids and the PFB ester derivatives, respectively. Extensive TLC was required in order to eliminate interfering compounds such as 5*S*,12*S*-diHETE. GC–NICI-MS analysis of the PFB-TMS derivatives in this study revealed LTB_4 serum concentrations of about 190 pg/ml whilst no LTB_4 could be detected in freshly collected human plasma. Five years later, Hughes and colleagues have measured by GC–NICI-MS LTB_4 concentrations of approximately 10 pg/ml in serum and about 5 pg/ml in plasma of normal volunteers when blood was collected into a tube containing the 5-LO inhibitor nordihydroguaiaretic acid (NDGA) [102]. In blood collected without NDGA, LTB_4 serum concentrations were 10 times higher indicating ex vivo formation of LTB_4. The assay of Hughes et al. was reported to exhibit an imprecision of 17% at a serum LTB_4 concentration of 11 pg/ml and an accuracy of 105% [102]. In a previous work, Hughes and colleagues have also measured serum LTB_4 levels of the order of 200 pg/ml by GC–NICI-MS–MS [175]. Ex vivo formation of LTB_4 but not interfering compounds could be the reason for these high, chemotactically active [261] LTB_4 serum levels [175,179]. Hughes et al. have used $[^2H_4]LTB_4$ as internal standard, SPE on C_{18} cartridges, straight-phase HPLC of the PFB esters for purification and GC–NICI-MS analysis of the PFB-*tert*-butyldimethylsilyl ether derivatives using SIM of m/z 431 and m/z 435 [102]. The straight-phase HPLC used satisfactorily separated the PFB ester of LTB_4 from the PFB esters of 6-trans-12-epi-LTB_4, 6-trans-LTB_4 and 5*S*,12*S*-LTB_4. These and other diHETEs may interfere with the quantitative GC–MS analysis of LTB_4 due to close retention times of their PFB-TMS derivatives on capillary columns [56]. In a mixture containing LTB_4 and its isomers 6-trans-12-epi-LTB_4, 6-trans-LTB_4 and 5*S*,12*S*-LTB_4, LTB_4 can only qualitatively be distinguished by CID of the parent ion m/z 479 since the major daughter ion of 6-trans-12-epi-LTB_4, 6-trans-LTB_4 and 5*S*,12*S*-LTB_4 is m/z 317, whilst under the same conditions the most intense daughter ion of LTB_4 is m/z 299 [69].

Dawson and colleagues have reported a GC–tandem MS method for quantification of LTB_4 in synovial fluid of patients with various arthropathies [207]. Their method involved a single SPE procedure on C_{18} cartridges and permitted quantitation by GC–NICI-MS–MS as less as 10 pg/ml. Quantitation by GC–NICI-MS was found to be not selective enough to allow quantification of LTB_4 in synovial fluid. By this method, LTB_4 could be detected at concentrations of 25 pg/ml and 1 ng/ml at an imprecision of 11.4% and 7.3%, respectively. The detection limit of the method was reported as 10 pg/ml [207]. We have recently shown the applicability of GC–NICI-MS–MS for the quantitative determination of LTB_4 in human plasma [113]. We have also shown in this work that the method is useful to simultaneously quantitate LTB_4 and LTE_4 in synovial fluid samples from arthritic patients, thus providing a methodology to assess total 5-LO activity in man within a single analysis (Fig. 11). Simultaneous measurement of LTB_4 and LTE_4 in synovial fluid required catalytical hydrogenation of LTB_4 to 5,12-dihydroxy-eicosanoic acid. This treatment enhanced sensitivity and specificity of the GC–NICI-MS–MS approach [113]. Because of significant ^2H-exchange during hydrogenation, $[1,2\text{-}^{13}C_2]LTB_4$ was used as internal standard. For measurement of LTB_4 in plasma by GC–NICI-MS–MS, a SPE on C_{18} cartridges and a lactonization procedure [262] without further purification steps were used [113].

GC–MS in the EI and NICI mode has been frequently applied to identify LTB_4 in urine, plasma, synovial fluid, lung lavage and other biological fluids of humans [69,253,254,257,261,263,264]. EI and NICI mass spectra of various derivatives of LTB_4 and its metabolites have frequently been reported [125,194,261,265–267].

4.3. HETEs, lipoxins, hepoxicilins and other eicosanoids

HETEs, lipoxins and hepoxicilins are stable biologically active metabolites of HPETEs [16–19,191]. GC–MS and LC–MS have played a key role in the structural identification and quantification of these eicosanoids and epoxyeicosatrienoic acids in vitro in

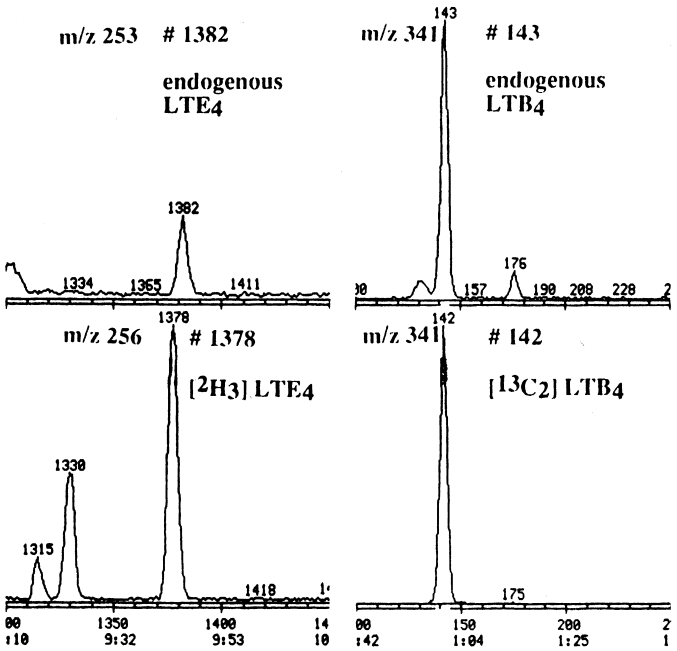


Fig. 11. Partial GC–NICI-MS–MS chromatograms from the simultaneous analysis of LTE_4 and LTB_4 in a human synovial fluid sample from an arthritic patient. [20,20,20-$^2H_3$]LTE_4 and [1,2-$^{13}C_2$]LTB_4 were used as internal standards. LTE_4 and LTB_4 were catalytically hydrogenated and converted to the PFB-TMS derivatives. From Ref. [113] with permission.

isolated cell systems and in cell-free systems [17,40–42,46,47,92,125,268–272]. On the other hand, only very little information is available about in vivo formation of these eicosanoids in humans most likely through their extensive metabolism analogous to LTB_4. Scientific interest in the in vivo formation of eicosanoids in humans other than prostanoids and leukotrienes has been almost exclusively focused on HETEs [74,166,176]. Walenga et al. reported a GC–EI-MS method for the quantitative determination of 5-, 12- and 15-HETE in human plasma and serum using 12-hydroxy-oleic acid as the internal standard [166]. These HETEs were found to be present in plasma of healthy humans at mean concentrations below 100 n*M*. HETEs were extracted from plasma by solvent extraction followed by SPE on silicic acid columns and separated by RP–HPLC. The presence of 12- or 15-HETE in urine of healthy or diseased humans has not been reported until 1996. In this year, Mayatepek and Lehmann have shown by GC–NICI-MS and RIA for the first time the occurrence of 12- and 15-HETE exclusively in the urine of patients with a peroxisome deficiency disorder, e.g. Zellweger syndrome, and found by RIA, mean concentrations of 26 and 40 pg/ml, respectively [74]. This study indicated that peroxisomes are the main cellular organelle responsible for HETEs oxidation in vivo in humans, analogous to leukotrienes and prostanoids [69,131]. 12- and 15-HETE were detected in urine of patients with Zellweger syndrome in their free acid forms. β-Glucuronidase treatment of urine samples had no influence on the urinary concentrations of these HETEs. Prakash et al. reported that 20-HETE is excreted as a glucuronide conjugate in human urine [176]. 20-HETE was quantitated in human urine by GC–NICI-MS using [20,20-$^2H_2$]20-HETE as the internal standard. Untreated and β-glucuronidase treated human urine samples were extracted on Chem-Elute columns. Sample purification was carried out by TLC and straight-phase HPLC of the PFB esters. Treatment of urine with β-glucuronidase resulted in a 13- to 28-

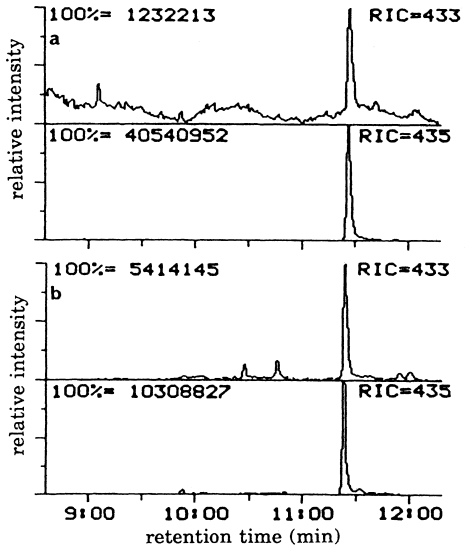


Fig. 12. Partial GC–NICI-MS chromatogram from the analysis of 20-HETE in a urine sample before (a) and after (b) β-glucuronidase treatment. Upper traces show endogenous 20-HETE and lower traces the internal standard [$^2H_2$]20-HETE as the PFB-*tert*-butyldimethylsilyl derivatives. From Ref. [176] with permission.

fold increase in 20-HETE concentration suggesting that 20-HETE is excreted primarily as a glucuronide conjugate (Fig. 12).

4.4. Urinary excretion rates of index metabolites in healthy humans

Urinary excretion rates of the most important index metabolites of prostanoids and leukotrienes by healthy humans reported in the literature, mainly in methodological works, are summarized in Table 3. The highest excretion rates have been measured for PGF-M and PGE-M which are substantially greater than that of 2,3-dinor-6-oxo-$PGF_{1\alpha}$, 2,3-dinor-TxB_2, 11-dehydro-TxB_2 or LTE_4.

5. Clinical applications

The implication of eicosanoids, particularly of prostaglandins, in various physiological and pathophysiological processes, and the discovery by Vane [273] that aspirin-like drugs produce their therapeutic and unwanted side-effects by inhibition of prostaglandin synthesis led to numerous investigations. The effects of such drugs and the role of eicosanoids in health and disease have frequently been reviewed [85,274–280]. The recent discovery of two different COXs [281,282], the constitutive COX-1 and the inducible COX-2, which provides an explanation for important differences among the nonsteroidal antiinflammatory drugs (NSAIDs), has led to the development of a new generation of NSAIDs with an improved side-effect profile and to a new classification of these drugs according to the relative inhibition of COX isoenzymes [283,284]. Among the NSAIDs, aspirin is the best investigated drug inhibiting prostanoid synthesis. In numerous clinical investigations on eicosanoid synthesis in vivo in humans under physiological and various pathophysiological conditions, during administration of aspirin and other NSAIDs or administration of dietary polyunsaturated fatty acids [288], and also in studying pharmacokinetics of PGE_1 (alprostadil) [289], GC–MS and GC–tandem MS methods were largely used to assess eicosanoids in human plasma and urine [69,74,83,123,131] [135,164,172,204, 222,223] [240,241,279,285,287,290–298]. The use of aspirin in low doses for primary and secondary prevention of myocardial infarction is based on the relatively selective inhibition of platelet aggregation and thromboxane formation [285–287]. By measuring 2,3-dinor-TxB_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in human urine by GC–NICI-MS–MS, Böger et al. have shown that intravenously administered low-dose aspirin inhibited TxB_2 synthesis within less than 2 h while sparing systemic COX activity; partial inhibition was found to be an unavoidable consequence of effective inhibition of platelet COX by aspirin (Fig. 13A) [287]. Stichtenoth et al. have found by measuring PGE-M and PGE_2 in human urine by GC–NICI-MS–MS that the new NSAID meloxicam is COX-1 sparing in humans in vivo unlike indomethacin (Fig. 13B) [296]. In accordance with the rare use of GC–MS methods for measurement of in vivo synthesis of leukotrienes in humans, GC–MS techniques have been applied in clinical studies almost exclusively to prostanoids.

Currently, the reliability of isoprostanes as markers of oxidative stress in vivo is feverishly investigated by several groups utilizing GC–MS

Table 3
Urinary excretion rates of important index metabolites of eicosanoids in healthy humans as measured by various methodological GC–MS methods

Eicosanoid	Excretion rate	Method	References
2,3-Dinor-6-oxo-$PGF_{1\alpha}$	719 ng/24 h	EI-MS	Falardeau et al. [154]
	233 ng/24 h	EI-MS	Vesterqvist and Gréen [164]
	74 pg/mg creatinine	NICI-MS	Chiabrando et al. [159]
	500 pg/mg creatinine	NICI-MS	Weber et al. [172]
	156 ng/24 h	NICI-MS–MS	Ferretti and Flanagan [184]
	141 pg/mg creatinine	NICI-MS	Daniel et al. [111]
	26–375 pg/ml	EI-MS	Mizugaki et al. [170]
	380 pg/mg creatinine	NICI-MS–MS	Fauler et al. [131]
PGE-M	17 μg/24 h	EI-MS	Hamberg and Samuelsson [118]
	14 μg/24 h	EI-MS	Ferretti et al. [167]
	6 ng/ml	NICI-MS–MS	Schweer et al. [150]
	18 ng/mg creatinine	NICI-MS–MS	Fauler et al. [131]
PGF-M	24 μg/24 h	EI-MS	Hamberg [136]
	14 ng/mg creatinine	EI-MS	Brash et al. [137]
	9 and 20 ng/ml	EI-MS	Ferretti et al. [195]
	7 ng/ml	NICI-MS–MS	Schweer et al. [150]
PGD-M	1.1 ng/mg creatinine	NICI-MS	Morrow et al. [135]
	0.8 ng/mg creatinine	NICI-MS	Awad et al. [109]
2,3-Dinor-TxB_2	138 pg/mg creatinine	NICI-MS	Lawson et al. [155]
	104 pg/mg creatinine	NICI-MS	Chiabrando et al. [157]
	530 ng/24 h	NICI-MS–MS	Schweer et al. [142]
	191 pg/mg creatinine	NICI-MS	Chiabrando et al. [159]
	21–266 pg/ml	NICI-MS–MS	Uedelhoven et al. [169]
	1050 pg/mg creatinine	NICI-MS	Weber et al. [172]
	360 pg/mg creatinine	NICI-MS–MS	Fauler et al. [131]
11-Dehydro-TxB_2	1200 ng/24 h	NICI-MS–MS	Schweer et al. [142]
	501 pg/mg creatinine	NICI-MS–MS	Lorenz et al. [171]
	47–942 pg/ml	NICI-MS–MS	Uedelhoven et al. [169]
	662 pg/mg creatinine	NICI-MS	Weber et al. [182]
	804 pg/mg creatinine	NICI-MS–MS	Ferretti et al. [183]
	370 pg/mg creatinine	NICI-MS	Morrow and Minton [11]
LTE_4	68 pg/ml	NICI-MS–MS	Tsikas et al. [129]
	31 nmol/mol creatinine	NICI-MS–MS	Tsikas et al. [132]
20-Carboxy-LTE_4	23 nmol/mol creatinine	NICI-MS–MS	Tsikas et al. [132]

Reported mean levels, ranges or single values are given in the original units.

methods for quantification of both COX-dependent prostanoids and isoprostanes in urine and plasma. These investigations imply studying the effect of COX-inhibiting drugs such as aspirin, diclofenac and indomethacin as well as of antioxidants such as vitamin E on the formation of isoprostanes, in particular of 8-iso-$PGF_{2\alpha}$. There are contradictory reports on this issue. Bachi et al. have found reduction of urinary 8-iso-$PGF_{2\alpha}$ during COX inhibition in rats but not in man [299]. They concluded that 8-iso-$PGF_{2\alpha}$ is a suitable marker of oxidative stress in man but not in rats. On the other hand, Schweer et al. have reported that 8-iso-$PGF_{2\alpha}$ is at least in part endogenously formed in humans via the

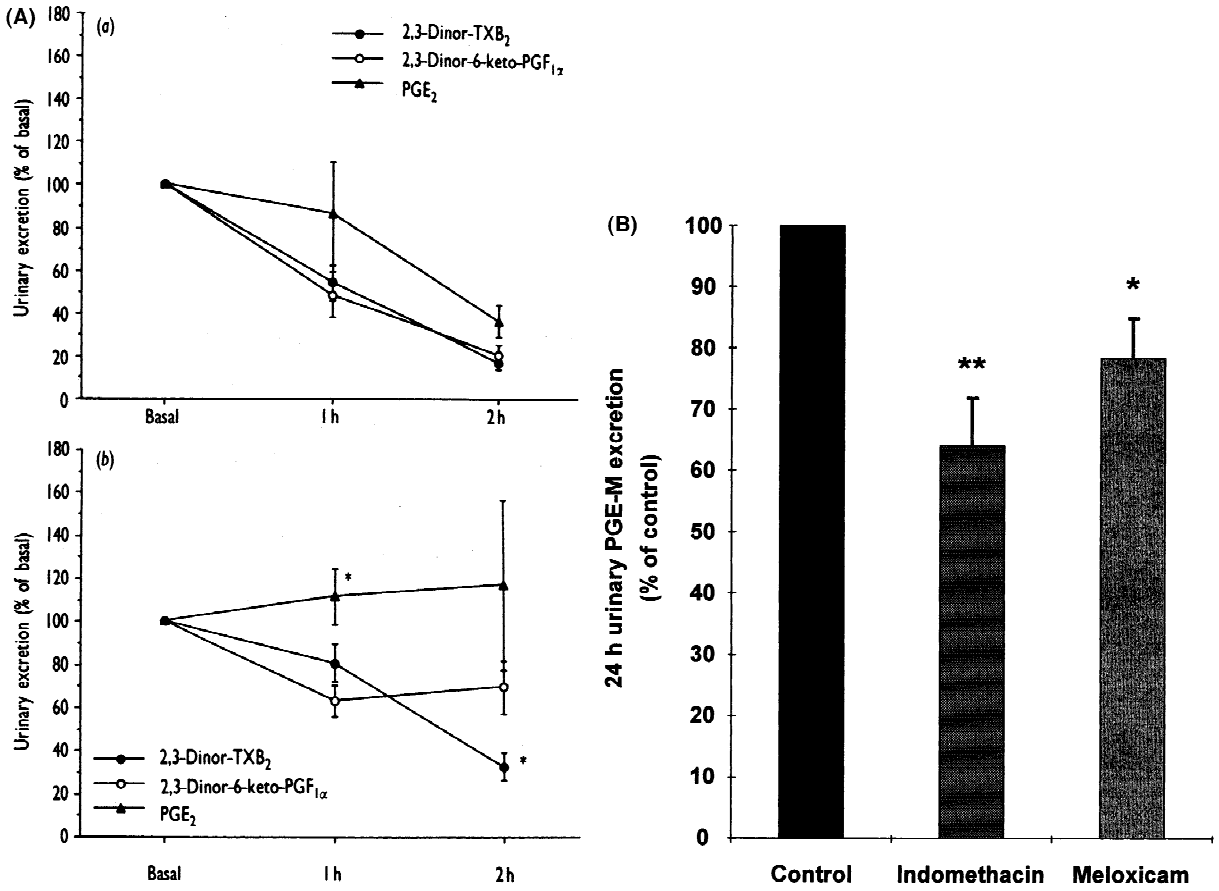


Fig. 13. (A) Urinary excretion of 2,3-dinor-TxB_2, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and PGE_2 before (basal), during (1 h) and after (2 h) the intravenous infusion of (a) 500 mg or (b) 50 mg of aspirin over 60 min. Basal prostanoid excretion was defined as 100% to correct for the different absolute levels of the three prostanoids in urine. Data points are means±SEM for $n=10$ subjects. Asterisks indicate significant differences between the treatment groups ($P<0.05$). With permission of [287]. (B) Effects of meloxicam at 7.5 mg per day and indomethacin at 25 mg 3 times a day on 24-h urinary PGE-M excretion. Mean (SE) of relative changes in percent (%) of control; $n=13$. *=$P<0.05$ vs control, **=$P<0.001$ vs control. From Ref. [296] with permission.

COXs [300]. This group has shown that urinary excretion of 8-iso-$PGF_{2\alpha}$ and of the classical prostanoids can be reduced after administration of indomethacin, but the suppression of 8-iso-$PGF_{2\alpha}$ was found to be less pronounced in comparison to COX-dependent prostanoids. Ferretti and Flanagan investigated the effect of lycopene, a carotenoid believed to have antioxidant capability in vivo, on the reduction of 8-iso-$PGF_{2\alpha}$ excretion by healthy volunteers [122]. This group reported that intake of 80 mg of lycopene per day for four weeks did not result in significant reduction of urinary excretion of 8-iso-$PGF_{2\alpha}$. We have recently found that chronic administration of L-arginine (2% in drinking water) or vitamin E (300 mg/day) to cholesterol-fed rabbits with preexisting hypercholesterolemia normalize urinary excretion of 8-iso-$PGF_{2\alpha}$ after twelve weeks [301]. By contrast, urinary excretion of 8-iso-$PGF_{2\alpha}$ by untreated hypercholesterolemic rabbits remained elevated for twelve weeks. These results indicate that vitamin E and L-arginine act antioxidatively in the rabbit, most likely via different mechanisms [301]. The significance of the quantification of F_2-isoprostanes to assess the role of oxidant injury in human diseases is recently reviewed by Morrow and Roberts [15,302].

6. Aspects of method validation and quality control

In general, validation of quantitation and quality control are indispenseable components of modern analytical methods. It is essential to use well-characterized and fully validated analytical methods to yield reliable results that can be satisfactorily interpreted. General aspects of analytical methods validation for the quantitative determination of drugs and their metabolites in biological samples and specific methodological aspects on quantitative mass spectrometry used for accuracy control in clinical chemistry have been reported in the past [303,304]. To our knowledge no equivalent reports appeared describing general directions for the quantitation of eicosanoids by GC–MS methods. Most of the GC–MS and GC–MS–MS methods cited and reviewed in this article appeared in analytical journals. The majority of these methods were thoroughly validated in accordance to generally accepted directions [303]. Important performance criteria of method validation of quantitative GC–MS methods based on ID–MS of eicosanoids included accuracy, precision, specificity, range of linearity and limit of detection [102,109–111,122,127,136,137,151,155,159,162,169,171,172,183,184,207]. Accuracy was mainly checked by supplementing the biological matrix with known amounts of the analyte to be quantitated and with a fixed amount of the internal standard. Accuracy of GC–MS and GC–MS–MS methods for the quantification of eicosanoids in human plasma and urine at basal levels is close to 100%. Because of the inherent accuracy of these methods, GC–MS has been used as a reference methodology for other methods such as RIA and EIA [52,56,78,104,105]. Precision was assessed by repeatedly determining analyte concentration in a matrix in different assay series, to take account of both intra- and inter-assay imprecision. The requisite precision is not necessarily the same at all analyte concentrations. Imprecision of GC–MS and GC–MS–MS methods for the quantification of eicosanoids at basal levels is below 10% in human urine and below 20% in human plasma. The sensitivity of an ID–MS procedure is of interest in characterising the ability of the method to discriminate small concentrations differences in the intended measurement range. The limit of detection of most GC–NICI-MS and GC–NICI-MS–MS methods is typically of the order of a few pg of an eicosanoid per ml of urine or plasma. Absolute recovery was commonly determined by using radiolabelled eicosanoids. Final absolute recovery which usually ranges between 20 and 90% depends on the number of the purification steps used. For example, Falardeau et al. have reported that the final organic extracts contained approximately 80% of the radioactive internal standards added to the urine samples [154]. When further purification by TLC was employed, the recovery of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ was approximately 50% after the chromatography [154]. The requirements of the internal standards and the problems associated with the impurity of the internal standard for the quantitation of eicosanoids by GC–MS methods are thoroughly discussed in the Section 3.2 of this article.

Unlike validation of quantitation, quality control in clinical studies, in which eicosanoids synthesis has been studied by quantifying index parameters in human urine and plasma by GC–MS methods, has been poorly and rarely performed [288]. This is not surprising if one considers that clinical studies are commonly performed by highly specialized investigators who used routine self-developed and validated GC–MS methods. Nevertheless, clinical studies in humans involving assessment of eicosanoid synthesis by GC–MS should imply performance of quality-control samples with regard to precision and accuracy in order to assure reliable results. With regard to the relatively high labor and the inherent accuracy of GC–MS methods, it is not necessary for quality control in methods for quantitation of eicosanoids in human urine and plasma by GC–MS to be performed to the same extent as it is usually required for drugs [303].

7. Conclusions and future prospects

From the beginning of eicosanoid research, GC–MS has played a key role not only in the structural identification of the numerous members of this large family but also in quantitatively assessing their formation in vivo in man. This is best managed by measuring specific index metabolites in urine. Dur-

ing the last two decades, a variety of GC–MS methods have been developed, improved and simplified for most primary eicosanoids and their index metabolites in urine and plasma. The development of sophisticated solvent and solid-phase extraction and of highly specific immunoaffinity extraction procedures has led to relatively simple and short analytical methods for many eicosanoids. Also, GC–MS–MS eliminated some time-consuming chromatographic steps. The introduction of NICI in quantitative analysis and improvements in instrumentation considerably reduced the volume of urine or plasma needed. Despite great improvements in GC–MS or GC–MS–MS, these techniques could not be automated to a high degree, unlike RIA and EIA methods. Also, for many eicosanoids lacking in specific physicochemical properties, extensive chromatography is still required. Nevertheless, GC–MS methods, GC–tandem MS being the ultimate reference method, are currently the most useful analytical tools for reliable routine quantitation of eicosanoid formation in vivo in humans.

GC–MS and GC–tandem MS will be indispensable analytical techniques in the development of new NSAIDs with improved side-effect profile for the next few years. Also, these techniques will be valuable for investigating formation, metabolism and appropriation of the isoprostanes and isoleukotrienes as specific endogenous markers of oxidative stress in humans. Already existing methods for COX-dependent prostanoids could be the basis for the development of GC–MS methods for the quantitation of isoprostanes and their metabolites in human urine and plasma. Measurement of isoprostanes may help to explore the role of free radicals in the pathogenesis of human disease and to provide a valuable approach to defining the clinical pharmacology of antioxidants.

In clinical studies on eicosanoids quality control and quality assurance have been so far neglected. With regard to the constantly increasing number of GC–MS users in eicosanoid research who have to establish published analytical methods in their group, and in consideration of the growing significance of quality control in analytical methods, quality control should be established in the field of eicosanoid analysis, despite the inherent accuracy of GC–MS and GC–tandem MS techniques.

8. List of abbreviations

BSTFA	*N*,*O*-bis(Trimethylsilyl)trifluoroacetamide
C_{18}	Octadecylsilica
CAD	Collision-activated dissociation
CID	Collision-induced dissociation
COX	Cyclooxygenase
DIPEA	*N*,*N*-diisopropylethylamine
EET	Epoxyeicosatrienoic acid
EI	Electron impact
EIA	Enzyme-immunoassay
FAB	Fast atom bombardment
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
GC–MS–MS	Gas chromatography–mass spectrometry–mass spectrometry
HEA	Hydroxyeicosanoic acid
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroxyperoxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
ID-MS	Isotope-dilution mass spectrometry
LC	Liquid chromatography
LO	Lipoxygenase
LT	Leukotriene
M	Molecular mass
Me	Methyl
MID	Multiple ion detection
MO	Methoxime
MOX	Methoxyamine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NICI	Negative-ion chemical ionization
P	Parent ion
PBA	Phenylboronic acid
PFB	Pentafluorobenzyl
PG	Prostaglandin
PGE-M	Prostaglandin E major urinary metabolite
PGI-M	Prostacyclin major urinary metabolite
RIA	Radioimmunoassay
SIM	Selected-ion monitoring
SPE	Solid-phase extraction
SRM	Selected-reaction monitoring
SSQ	Single-stage quadrupole

TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TMSOH	Trimethylsilanol
TSQ	Triple-stage quadrupole
Tx	Thromboxane
Tx-M	Thromboxane major urinary metabolite
UV	Ultraviolet

Acknowledgements

The excellent laboratory assistance of M. Böhme and F.-M. Gutzki is gratefully acknowledged. The author thanks Dr. D.O. Stichtenoth for reading the manuscript and for his valuable comments on this work. Experiments carried out by the author and his group were supported by grants from the Deutsche Forschungsgemeinschaft (Fr 366) and the Bundesministerium für Forschung und Technologie (01VM90020).

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